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(54) Title: CORTICOTROPIN RELEASING FACTOR RECEPTOR 2 DEFICIENT MICE AND USES THEREOF

(57) Abstract: The present invention provides transgenic mice deficient in corticotropin releasing factor receptor 2. CRFR2 mutant mice are hypersensitive to stress, and display increased anxiety-like behavior. Mutant mice had normal basal feeding and weight gain, but exhibited decreased food intake following food deprivation. Intravenous UCN has no effect on the mutant mice, but causes decreased arterial pressure in control animals. Deficiency in CRFR2 results in a significant increase in urocortin mRNA in the rostral region of the Edinger Westphal, and a significant increase in CRF mRNA in the central nucleus of the amygdala. These results demonstrate that CRFR2 mutant mice, as opposed to CRFR1 mutant mice, have an increased sensitivity to stress and display anxiety-like behavior.

CORTICOTROPIN RELEASING FACTOR RECEPTOR 2

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DEFICIENT MICE AND USES THEREOF

BACKGROUND OF THE INVENTION

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Federal Funding Legend

Field of the Invention

The present invention relates generally to the fields of neurobiology, endocrinology, and psychiatry. More specifically,

20   the present invention relates to the study of anxiety and to mice deficient for corticotropin releasing factor receptor 2.

Description of the Related Art

Corticotropin releasing factor (CRF) is a critical

25   coordinator of the hypothalamic-pituitary-adrenal (HPA) axis. In response to stress, corticotropin releasing factor released from the paraventricular nucleus of the hypothalamus (PVN) activates corticotropin releasing factor receptors on anterior pituitary corticotropes, resulting in release of adrenocorticotropic hormone

(ACTH) into the bloodstream.   ACTH in turn activates ACTH receptors in the adrenal cortex to increase synthesis and release of glucocorticoids (1).

5   The receptors for CRF, CRFR1 and CRFR2 are localized throughout the CNS and periphery.   While CRF has a higher affinity for CRFR1 than for CRFR2, urocortin (UCN), a CRF-related peptide, is thought to be the endogenous ligand for CRFR2 since it binds with almost 40-fold higher affinity than does CRF (2). CRFR1 and CRFR2 share approximately 71% amino acid sequence

10   similarity and are distinct in their localization within the brain and peripheral tissues (3-6).   CRFR1 is expressed mainly in the pituitary gland, cortex, cerebellum, hindbrain, and olfactory bulb, whereas CRFR2 is found in the lateral septum, ventral medial hypothalamus (VMH), choroid plexus, and many peripheral sites

15   (5, 7, 8).   CRFR2 has several isoforms, one of which has been shown to not bind any known ligand (9).

Mice deficient for CRFR1 have decreased HPA axis hormone levels, an impaired stress response, and decreased anxiety-like behavior (10, 11). These results coincide with those

20   obtained using CRFR1 specific antagonists *in vivo* (12-14). In contrast, CRFR2 specific antagonists are not currently available, and since its cloning in 1995, little has been elucidated regarding the physiological function of CRFR2. UCN may be the endogenous ligand for CRFR2 and has been shown to be a modulator of feeding

25   when administered centrally (15).   Since CRFR2 is localized to the ventral medial hypothalamus, a central site of food intake regulation and satiety, it is possible that urocortin actions on these receptors   may   affect   feeding.   Further,   peripheral administration   of   urocortin results in hypotension (2,

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16) which may result from the action of CRFR2 in the vasculature
(5, 8).

The prior art is deficient in the lack of mice deficient for corticotropin releasing factor receptor 2. The present invention fulfills this longstanding need and desire in the art.

SUMMARY OF THE INVENTION

In order to discern the developmental and physiological roles of CRFR2, CRFR2 null mutant mice were generated and analyzed. CRFR2 deficient mice exhibit increased anxiety-like behavior and a hypersensitive HPA axis in response to stress. CRFR1 and CRFR2 mutant mice provide valuable models of anxiety and depression and may further help delineate the molecular mechanisms underlying these diseases. Study of the corticotropin releasing factor signaling pathway and its role in the management of anxiety and depression may provide the necessary clues required for the effective treatment of these diseases.

Thus, the present invention is directed to a non-natural transgenic mouse with a disruption in at least one allele of the corticotropin releasing factor receptor 2 (CRFR2) such that said mouse does not express corticotropin releasing factor receptor 2 protein from said allele. Preferably, the DNA sequences for exons 10, 11, and 12 of said corticotropin releasing factor receptor 2 allele have been deleted. The transgenic mouse may have these DNA sequences replaced with a neomycin resistance gene cassette. The transgenic mouse may be either heterozygous

or homozygous for this replacement. Also included in an embodiment of the present invention are the progeny of a mating between a mouse of the present invention and a mouse of another strain.

5   Another embodiment of the present invention is the application of a CRFR2 deficient mouse to the study anxiety or depression and to test the effects of a compound on anxiety or depression. For example, a method is provided of screening a compound for anxiety modulating activity, comprising the steps of:

10   a) administering said compound to the transgenic mouse of the present invention; b) testing said mouse for anxiety-related behavior; and c) comparing anxiety-like behavior of said mouse with anxiety-like behavior in a second transgenic mouse of the present invention to which said compound was not administered.

15   In addition, a method of screening a compound for depression-modulating activity is provided, comprising the steps of: a) administering said compound to the transgenic mouse of the present invention; b) testing said mouse for depression-like behavior; and c) comparing depression-like behavior of said

20   mouse with depression-like behavior in a second transgenic mouse of the present invention to which said compound was not administered.

         Yet another embodiment of the present involves the use of a CRFR2 deficient mouse in a similar procedure to screen for

25   compounds which affect blood pressure or angiogenesis.

         A further embodiment of the current invention is the application of the CRFR2 deficient mice to the study of the physiology of the HPA axis, e.g., a method of screening a compound for effects on the response of the hypothalamic-

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pituitary-adrenal axis to stress, comprising the steps of: a)
administering said compound to a transgenic mouse of the present
invention; b) placing said mouse in a stress-inducing situation; c)
monitoring plasma levels of corticosterone and adrenocorticotropic

5   hormone in said mouse; and d) comparing said levels to those in a
transgenic mouse of the present invention not placed in said
stress-inducing situation.

In yet another embodiment of the current invention,
the mice can be used to study the effects of a compound on the

10   response of the HPA axis to stress by monitoring plasma levels of
corticosterone and ACTH.

Yet another embodiment of the current invention
relates to the use of the mice of the instant invention in the study
the effect of corticotropin releasing factor receptor 2 on other

15   proteins such as corticotropin releasing factor and urocortin.

A further embodiment of the current invention is the
use of the CRFR2 deficient mice to examine CRFR1 responses
unhindered by the presence of CRFR2.

Yet another embodiment of the instant invention is the

20   manipulation of CRFR2 activity to stimulate or inhibit
vascularization.

BRIEF DESCRIPTION OF THE DRAWINGS

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So that the matter in which the above-recited features,
advantages and objects of the invention, as well as others which
will become clear, are attained and can be understood in
detail, more particular descriptions of the invention

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briefly summarized above may be had by reference to certain embodiments thereof which are illustrated in the appended drawings. These drawings form a part of the specification. It is to be noted, however, that the appended drawings illustrate

5   preferred embodiments of the invention and therefore are not to be considered limiting in their scope.

Figures 1A-1E show the procedure used for the generation of CRFR2-Deficient Mice, the detection of mutant mice, and the effects therein. Figure 1A is a representation of the

10   genomic organization of the CRFR2 gene showing the deletion of exons 10, 11, and 12 which code for half of transmembrane domain five through the end of transmembrane domain seven. The targeting construct utilized for homologous recombination is also shown. In Figure 1B, the disrupted allele was detected by

15   Southern Blot analysis of tail DNA isolated from wild type (+/+), heterozygote (+/-), and null mutant (-/-) mice. Figure 1C presents the results of autoradiographic binding of ^{125}I-Sauvagine in CRFR2 control (top) and mutant (bottom) mice. Note, no CRFR2 binding in the lateral septum of CRFR2 mutant mice, while the

20   CRFR1 cortical binding is similar to that of the control mouse. Figure 1D shows hematoxylin and eosin (H&E) staining of the adrenal glands. Note no difference in adrenal gland size (upper panels) at 10X magnification or structure (lower panels) at 20X magnification, C, cortex; M, medulla; ZG, zona glomerulosa; ZF, zona

25   fasciculata; ZR, zona reticularis; n=8. Figure 1E shows H&E staining of the pituitary glands which were mounted on liver for tissue sectioning (upper panels) at 4X magnification, n=8. Pituitary corticotropes were identified with anti-ACTH antibodies (20) (lower panels) at 10X magnification, n=5.

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P, posterior lobe; I, intermediate lobe; A, anterior lobe. No gross anatomical differences were observed for the pituitary gland or for the corticotrope localization or expression levels of ACTH.

Figures 2A-2D demonstrate the hypersensitivity of
5   the HPA axis to stress in mutant animals. * = significantly different from wild type controls at same time point, p< 0.01 by Scheffe post-hoc test. Plasma obtained by unanesthetized retro-orbital eyebleeds. Figure 2A shows pre-stress ACTH plasma levels at 7:00 AM, n=16. Figure 2B shows basal corticosterone
10   plasma levels for 7:00 AM and 5:00 PM, n=7. Figure 2C presents a time course of restraint stress effects on ACTH. Figure 2D demonstrates that corticosterone plasma levels at 7:00 AM are significantly different in null mutant mice from wild type controls at same time point, n=7.

15   Figures 3A-3B show the effects of 24 hours of food deprivation on food intake in wild type and mutant littermate mice. Figure 3A shows food consumption of mutant mice (n=7) basal and following a 24 hr food deprivation period as compared to wild type litter mates (n=10), p < 0.001 by Scheffe post-hoc test.
20   Figure 3B shows the weight of wild type and mutant mice, both the basal (open bars) weights and following 24 hrs of refeeding (black bars) following the food deprivation period. Note that there are no differences between the groups in basal or refed body weights.

25   Figures 4A-4H demonstrate the increased anxiety-like behavior of mutant animals in the elevated plus maze and open field test. Figure 4A shows that in male mice the percentage of time spent   in the open arms (**, p<0.005) and number of visits to the   open arms (*, p<0.02) were

significantly less for the mutant mice than for the wild type controls (control n=7, mutant n=7; mean ±SEM). **Figure 4B** shows the same test as Figure 4A for female mice. The percentage of time spent in the open arms (**, p<0.03) and number of visits to
5   the open arms (*, p<0.03) were significantly less for the mutant mice than for the wild type controls (control n=9, mutant n=12; mean ±SEM). **Figures 4C** and **4D** show that locomotor activity was not different between control and mutant animals (**Figure 4C**, male mice; **Figure 4D**, female mice) as measured by total
10 closed arm entries and total arm entries. **Figure 4E** shows that no differences were found in anxiety-like behavior measured in the light/dark box experiment for time spent in light portion of the box. **Figure 4F** shows that no differences were found in anxiety-like behavior measured in the light/dark box experiment
15 for the number of transitions between the light and dark portions. **Figure 4G** shows the amount of time spent in the inner squares of the open field apparatus (*, p<0.05). **Figure 4H** shows the present of total crossings occurring in the inner squares (**, p<0.01; controls, n=5; mutants, n=7; mean ±SEM).

20   **Figures 5A-5E** show increased levels of urocortin and CRF mRNA in the mutant brains. For 5B to 5E, all numbers were the average of n=3 for mutant and wild type mice, ±SEM, *, p < .05; ** p < .01; ***, p < .005, by Fischer's PLSD post-hoc test. **Figure 5A** shows silver grains resulting from *in situ*
25 hybridization for urocortin mRNA in the rostral EW (upper) at 20X magnification and CRF mRNA in cAmyg (middle) and paraventricular nucleus (lower) at 10X magnification. **Figure 5B** presents the semi- quantitative analysis of silver grains used to determine cell numbers expressing urocortin mRNA

in the rostral EW. **Figure 5C** shows the average optical density of urocortin mRNA per cell. **Figure 5D** shows the optical density of CRF mRNA in the cAmyg. **Figure 5E** shows the optical density of CRF mRNA in the paraventricular nucleus.

5   **Figure 6** shows cardiovascular responses to intravenous infusion of 1.0 μg urocortin in wild type (n = 5) and mutant mice (n = 3) (black bars). Note the remarkable muted response of mutant mice to the urocortin injection. *** $p < 0.005$. CRFR2 mutant mice also received a second infusion of sodium

10   nitroprusside (0.8 μg in 100 μl of 0.9% saline) following recovery of arterial pressure from the UCN infusion (open bar). The mean arterial pressure (MAP) was determined from the blood pressure tracings.

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DETAILED DESCRIPTION OF THE INVENTION

          In accordance with the present invention there may be

20   employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II

25   (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" [B. D. Hames & S.J. Higgins Eds. (1985)]; "Transcription and Translation" [B. D. Hames & S.J. Higgins Eds. (1984)]; "Animal Cell   Culture" [R. I. Freshney, ed. (1986)]; "Immobilized   Cells And   Enzymes" [IRL Press, (1986)]; B.

Perbal, "A Practical Guide To Molecular Cloning" (1984).

Therefore, if appearing herein, the following terms shall have the definitions set out below.

As used herein, the term "cDNA" shall refer to the DNA
5   copy of the mRNA transcript of a gene.

As used herein the term "screening a library" shall refer to the process of using a labeled probe to check whether, under the appropriate conditions, there is a sequence complementary to the probe present in a particular DNA library.
10   In addition, "screening a library" could be performed by PCR.

As used herein, the term "PCR" refers to the polymerase chain reaction that is the subject of U.S. Patent Nos. 4,683,195 and 4,683,202 to Mullis, as well as other improvements now known in the art.

15   The amino acids described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of immunoglobulin-binding is retained by the polypeptide. NH_2 refers to the free amino group
20   present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243:3552-59 (1969), abbreviations for amino acid residues are known in the art.

25   It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-   terminus. Furthermore, it should be noted that a dash at   the beginning or end of an amino

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acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

A "replicon" is any genetic element (e.g., plasmid, chromosome, virus) that functions as an autonomous unit of DNA replication *in vivo*; i.e., capable of replication under its own control.

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in its either single stranded form, or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, *inter alia*, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the nontranscribed strand of DNA (i.e., the strand having a sequence homologous to the mRNA).

An "origin of replication" refers to those DNA sequences that participate in DNA synthesis.

A DNA "coding sequence" is a double-stranded DNA sequence which is transcribed and translated into a polypeptide *in vivo* when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA

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from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

5   Transcriptional and translational control sequences are DNA regulatory sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for the expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region
10   capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum
15   number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eukaryotic promoters often, but
20   not always, contain "TATA" boxes and "CAT" boxes. Prokaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

An "expression control sequence" is a DNA sequence that controls and regulates the transcription and translation of
25   another DNA sequence. A coding sequence is "under the control" of transcriptional and translational control sequences in a cell when RNA polymerase transcribes the coding sequence into mRNA, which is then translated into the protein encoded by the coding sequence.

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A "signal sequence" can be included near the coding sequence. This sequence encodes a signal peptide N-terminal to the polypeptide that communicates to the host cell to direct the polypeptide to the cell surface or secrete the polypeptide into the media, and this signal peptide is clipped off by the host cell before the protein leaves the cell. Signal sequences can be found associated with a variety of proteins native to prokaryotes and eukaryotes.

The term "oligonucleotide", as used herein in referring to the probe of the present invention, is defined as a molecule comprised of two or more ribonucleotides, preferably more than three. Its exact size will depend upon many factors which, in turn, depend upon the ultimate function and use of the oligonucleotide.

The term "primer" as used herein refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension product, which is complementary to a nucleic acid strand, is induced, i.e., in the presence of nucleotides and an inducing agent such as a DNA polymerase and at a suitable temperature and pH. The primer may be either single-stranded or double-stranded and must be sufficiently long to prime the synthesis of the desired extension product in the presence of the inducing agent. The exact length of the primer will depend upon many factors, including temperature, source of primer and use the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain fewer nucleotides.

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The primers herein are selected to be "substantially" complementary to different strands of a particular target DNA sequence. This means that the primers must be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5' end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementary with the sequence or hybridize therewith and thereby form the template for the synthesis of the extension product.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells

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derived from a single cell or ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

5   In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted DNA fragment are used in connection with the host. The expression vector typically contains an origin of replication, promoter(s), terminator(s), as well as specific genes which are capable of providing phenotypic selection in transformed cells.

10   The transformed hosts can be fermented and cultured according to means known in the art to achieve optimal cell growth.

          Methods well known to those skilled in the art can be used to construct expression vectors containing appropriate transcriptional and translational control signals. See for example,

15   the techniques described in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual* (2nd Ed.), Cold Spring Harbor Press, N.Y. A gene and its transcription control sequences are defined as being "operably linked" if the transcription control sequences effectively control the transcription of the gene. Vectors of the

20   invention include, but are not limited to, plasmid vectors and viral vectors.

          The current invention is directed to mice deficient for CRFR2, which were generated to discern the developmental and physiological roles of CRFR2 in anxiety and HPA axis circuitry.

25   This was done by deleting exons 10, 11, and 12 of corticotropin releasing factor receptor 2. In the present invention, these sequences have been replaced with a neomycin resistance gene cassette. The mice may be either heterozygous of homozygous for the CRFR2 deficiency and may be crossed

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with mice of another strain.

The present invention is also directed to the application of the CRFR2 deficient mice in the study of anxiety and depression, including methods of testing a compound for anxiety or depression modulating activity. Compounds which affect blood pressure and angiogenesis can also be screened using the CRFR2 mice.

The current invention is also directed to use of the CRFR2 deficient mice in the study of the molecular physiology of the hypothalamic-pituitary-adrenal (HPA) axis. The mice can be used to test the effects of a compound on the response of the HPA axis to stress.

The current invention is also directed to the use of the transgenic mice to study the molecular functions of corticotropin releasing factor receptor 2 on corticotropin releasing factor, corticotropin releasing factor receptor 1, urocortin, and other CRF and urocortin receptors.

In addition, the present invention can be used to study the responses and activities of CRFR1 in a CRFR2 negative environment. In this manner, CRFR1 responses can be studied unhindered by CRFR2 modulation.

The instant invention is also directed the use of agonist or antagonist of CRFR2 activity to stimulate or inhibit vascularization. The use of agonist to stimulate CRFR2 activity may be useful for the inhibition of vascularization for the treatment of cancer.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

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EXAMPLE 1

Generation of the CRFR2 Deficient Mice

        For the construction of CRFR2 null mutant mice, a
5   genomic clone DNA containing the *CRFR2* locus was isolated from a
mouse strain 129 genomic DNA library. From this clone, a
targeting vector was constructed in which the exons 10, 11, and
12 of the *CRFR2* gene encoding the beginning of the fifth
transmembrane domain through the end of the seventh
10 transmembrane domain were replaced with a neomycin resistance
gene cassette (Figure 1A). The resulting plasmid DNA was
linearized with Not I and electroporated into J1 embryonic stem
(ES) cells as previously described (10). After selection in 0.2
mg/ml G418 (active form) for 7-9 days, neomycin resistant clones
15 were individually selected and screened for the presence of the
disrupted CRFR2 allele by Southern blot analysis.

        Positive ES clones were injected into C57BL/6
blastocysts to generate chimeric mice. Chimeric males were
crossed to chimeric females producing C57BL/6-129 mixed
20 background mice. Germ-line transmission of the disrupted allele
was detected by Southern analysis of tail DNA collected from F1
pups displaying agouti coat color (Figure 1B).

EXAMPLE 2

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Analysis of CRFR1 and CRFR2 expression in CRFR2 Deficient Mice

        To determine if the targeted deletion resulted in a null
mutation of the CRFR2 gene, receptor autoradiography
was performed on brain sections from wild type control and

mutant animals.

Slides containing 20 μm sectioned brain tissue were thawed at room temperature and washed twice for 10 min. in 50 mM Tris buffer (pH 7.4) at room temperature. Sections were then incubated in buffer containing 50 mM Tris (pH 7.4), ^{125}I-Sauvagine, 10 mM MgCl$_2$, 0.1% BSA, and 0.05% bacitracin for 60 min. at room temperature. Sauvagine is a CRF-related peptide and an agonist at the CRF receptors. Nonspecific binding was defined in adjacent sections which were exposed to both ^{125}I-Sauvagine and 1 μm cold sauvagine. After the incubation period, slides were washed in a 50 mM Tris buffer plus 0.01% Triton X-100 at 4°C twice for 5 min. each. Slides were rapidly dipped in deionized water, dried and apposed to film for 3 days.

In the mutant mice, no binding in brain regions specific to CRFR2 (lateral septum) was detected, yet binding to CRFR1 in the cortex was retained (Figure 1C). These results demonstrate that the disruption of the CRFR2 gene resulted in a null mutation in these mice. Mutant mice were fertile and transmitted the mutant allele in a Mendelian fashion.

EXAMPLE 3

Histological analysis of CRFR2 Deficient Mice

To determine whether the development of the HPA axis was compromised in the CRFR2 deficient mice, the pituitary and adrenal glands of male mice 10-12 week of age were sectioned and stained with hematoxylin and eosin (H&E). Briefly, mice were perfused with 4% paraformaldehyde (PFA). Tissues were removed, postfixed overnight at 4 C, and

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cryoprotected in 30% sucrose in PBS. Tissues were sectioned at 1 2 μm thickness and stained with hematoxylin and eosin. The results showed no obvious differences in structure or cell types (Figs. 1D-1E).

5   In addition, pituitary sections were stained with anti-ACTH antibodies. The pituitaries were sectioned, postfixed in 4% PFA for 5 min., rinsed in PBS, and stained with ACTH antibody a s described previously (10). No qualitative differences were noted between wild type and mutant corticotropes (Fig. 1E).

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EXAMPLE 4

Determination of corticosterone and ACTH levels in CRFR2 deficient mice

15   For corticosterone and ACTH analyses, plasma was obtained from individually housed male mice of 10-12 weeks of age. Samples were collected by retro-orbital eye bleed from unanesthetized animals within 30 sec of disturbance of the cage. Basal AM samples were collected at 7:00 AM. Basal PM samples

20   were collected at 5:00 PM. Corticosterone assay (ICN Biomedicals, Dosta Mesa, CA) used 5 μl plasma and the ACTH assay (Nichols Institute Diagnostics, San Juan Capistrano, CA) used 50 μl plasma as measured in duplicate by radioimmune assay kits. Normal basal levels of ACTH and corticosterone were found in the mutant

25   and control animals (Fig. 2A-2B), consistent with the finding in Figure 1E that ACTH levels are unaffected in the brain.

EXAMPLE 5

Effects of stress on the HPA axis response in CRFR2 deficient Mice

In order to examine the HPA axis response to stress,
5   animals were subjected to physical restraint-stress for increasing
lengths of time. Blood samples were collected immediately
following either 2, 5, or 10 min. of restraint stress in a 50 ml
conical tube (plastic conical tube with the bottom removed). Each
mouse was bled only once. Plasma samples were immediately
10 centrifuged and stored at -20C until the assay was conducted. The
Corticosterone assay (ICN Biomedicals, Costa Mesa, CA) used 5 µl
plasma and the ACTH assay (Nichols Institute Diagnostics, San Juan
Capistrano, CA) used 50 µl plasma as measured in duplicate by
radioimmune assay kits.
15   ACTH levels in control animals peaked following ten
minutes of restraint. In contrast, ACTH levels in the mutant
animals were significantly elevated and peaked following only two
minutes of restraint stress (Fig. 2C). Similarly, corticosterone
levels in the mutant animals were significantly elevated following
20 two minutes of restraint, whereas control animal levels increased
following five minutes of the stress (Fig. 2D). These results
demonstrated a hypersensitive response of the HPA axis to stress
in the mutant mice.

25   EXAMPLE 6

CRFR2 deficient mice are sensitive to food deprivation

Since CRFR2 is   abundant in the VMH and since
previous   studies   had   shown   an   anorectic   effect   of

urocortin (15), basal feeding and weight gain were measured in the mutant and wild type littermates.

Basal feeding was measured in individually housed 12-16 week old male littermates. Mice and their food pellet were
5   weighed daily at 09:00 hrs. For the food deprivation experiment, control and mutant litter mates were individually housed and their basal food intake and weight was established. Mice were food deprived for 24 hrs beginning at 12:00 hrs, but had water ad libidum. Following the food deprivation period, mice were
10   weighed and given a pre-weighed food pellet. Food pellets were then weighed every two hours until lights off (18:00 hrs). Food pellets and mice were again weighed the following morning. Weight loss during the food deprivation, as well as total food consumption and weight gain over the 24 hr period following the
15   food deprivation, were recorded. Basal feeding and weight gain in CRFR2 mutant (mut) male mice were similar to that of wild type (wt) litter mates (24 hr basal food consumption wt = 4.3 $\pm$ 0.24 g, mut = 4.6 $\pm$ 0.23 g; body weight wt = 21.7 $\pm$ 0.66 g, mut = 21.2 $\pm$ 0.50 g; n=10, averages are $^+_-$ SEM).

20   In order to determine if a stressful stimulus would alter the mutant animals' food intake, control and mutant mice were food deprived for 24 hrs and then refed, following which their food intake and weight changes were measured. Food deprivation results showed a significant decrease in food intake in
25   the mutant mice following 24 hrs of food deprivation (Fig. 3A). Mutant mice consumed 75% of wild type food levels in the 24 hr period following the food deprivation. However, the mutant and wild type body weights were not significantly different following food deprivation or refeeding (Fig. 3B).

21

EXAMPLE 7

Evaluation of anxiety-like behavior in CRFR2 deficient mice in Elevated Plus Maze

Since CRFR1 mutant mice displayed anxiolytic-like behavior (10), CRFR2 mutant mice were analyzed in similar tests using three different test paradigms. In the first test paradigm, control and mutant animals were evaluated using the elevated plus maze (EPM). Male and female mice between 22-24 weeks of age were used in this experiment. Wild type littermate mice were used as the controls. Animals were group housed, maintained under regular light/dark conditions (lights on 6:00 AM, lights off 6:00 PM), and handled on alternate days one week prior to testing.

The plus maze apparatus was made of black Plexiglas and had two open arms (30 x 5 cm) and two enclosed arms of the same size with walls 30 cm high. It was elevated 30 cm above the ground. The arms were connected by a central square (5x5 cm) and thus the maze formed a plus sign. A 25 watt lamp placed above the apparatus provided a 6 lux light level in the open arms. All testing was performed during the light phase of the light-dark cycle. Mice were habituated to the experimental room conditions for 1 hour prior to the behavioral testing and the subjects were individually tested in 5-min sessions.

Each mouse was placed on the center platform facing an open arm to initiate the test session. Behaviors scored were the number of open and closed arm entries and the amount of time spent on the various sections of the maze. Arm entries were defined as an entry of all four paws into the arm.

Closed arm entries were taken as an index of locomotor activity in the plus maze. A camera mounted above the apparatus allowed the observation of animal behavior on a video monitor placed in an adjacent room. At the end of the test, the number of entries into and the time spent on the open arms were expressed as a percentage of the total number of arm entries and test duration, respectively. Results are expressed as the mean $\pm$ standard error of the mean. Behavioral parameters obtained from the EPM test were analyzed using the Student's t test.

Results showed that both male and female CRFR2 mutant mice spent less time on and entered less frequently the open arms of the plus-maze apparatus than did the wild type controls. A significant effect was found for both percent entries into the open arms for both male and female mice (Figures 4A and 4B). The increase in anxiety-like behavior was not due to altered locomotor activity, as overall activity in closed arm and total arm entries was not different between the two groups (Figs. 4C and 4D). These results demonstrate that CRFR2 mutant mice exhibit markedly increased anxiety-like behavior.

EXAMPLE 8

Evaluation of anxiety-like behavior in CRFR2 deficient mice in a light/dark box

The behavior of CRFR2 mutant and control mice was also analyzed for anxiety-like behavior in a light/dark box. A rectangular, plexiglass box was divided into two compartments, one painted white (28.5 cm x 27 cm) and one painted black (14.5 cm x 27.0 cm). Light intensity was 8 lux in the

23

black compartment covered by a red plexiglass lid and 400 lux in the white compartment. The compartments were connected by an opening (7.5 cm x 7.5 cm) located at floor level in the center of the partition. All testing was done during the dark phase of the cycle,

5   between 19:00 hrs and 21:00 hrs. Each animal was tested for 10 min by being placed in the center of the white area and the number of transitions between the two compartments and the amount of time spent in the white area was recorded. A camera mounted above the apparatus allowed for observation and

10   recording from an adjacent room.

Results from the Light/Dark box demonstrated that CRFR2 mutant mice spent as much time in the light portion of the box and had as many transitions between the light and dark portions of the box as control mice (Fig. 4E&4F). No significant

15   differences were detected between the two groups in this experiment.

EXAMPLE 9

Evaluation of anxiety-like behavior in CRFR2 deficient mice in an
20   open field test

Anxiety-like behavior was also analyzed in CRFR2 mutant and control mice in an open field apparatus. The open-field apparatus consisted of a white plexiglass box (50 x 50 x 22 cm) with 16 squares (12 x12 cm) painted on the floor (12 outer

25   and 4 inner). A lamp directed to the center of the field provided a 120 lux illumination on the floor. Testing was conducted during the dark phase of the light-dark cycle in a room with constant background   white   noise   (52 dB). Each mouse was placed in the   center   of   the   apparatus to initiate a 10-min test

session.   Time (sec) spent in the inner squares, ambulation (number of squares crossed), defecation (number of fecal boli), rearings, and time spent grooming (sec) were quantified from a video recording. Inner square crossings were also expressed as a percentage of ambulation. The results from the open-field test showed that the CRFR2 mutant mice spent less time in and displayed a lower percentage of inner square crosses than did wild type mice (Figs. 4G&H). No differences in ambulation, rearings, defecation, or grooming were observed between the groups (data not shown).

EXAMPLE 10

Effect of CRFR2 deficiency on the expression of other genes

As no gross anatomical defects were detected in components of the HPA axis (Figures 1D & 1E), the alterations in stress and behavioral responses in the mutant animals may be due to altered gene expression of other components of the CRF signaling pathway. To investigate this possibility, expression of UCN, CRF, and CRFR1 mRNAs were examined by *in situ* hybridization.

In situ hybridization was performed according to methods described previously (36). Briefly, tissue sections (20 μm) were fixed in 4% paraformaldehyde, rinsed in PBS, immersed in acetic anhydride, dehydrated through a series of graded ethanol, de-lipidated in chloroform, and again dehydrated. Slides were then hybridized with an ^{35}S-labeled riboprobe in a 50% deionized formamide hybridization mix overnight at 55°C in a humidified incubation chamber. Following the incubation, slides were washed in 1X SSC at room temperature for 30 minutes

with shaking, treated with 20 μg/ml RNase (Promega) at 37 C for 30 min., rinsed in 1X SSC buffer at room temperature for 3 0 minutes, washed 3X for 20 minutes at 65 C in 0.1X SSC with shaking, rinsed in 0.1X SSC at room temperature for 30 minutes,

5   dehydrated in a series of graded ethanols, air dried, and apposed to Kodak hyperfilm (Eastman Kodak, Rochester, NY) for three days.

After films were developed, slides were dipped in NTB2 liquid nuclear emulsion (Eastman Kodak; diluted 1:1 with water), exposed for 10 days, photographically processed, counter-

10   stained with hematoxylin, and coverslipped. Slides were analyzed using the image analysis system Image Pro Plus (Media Cybernetics, Silver Springs, MD). For analysis of the PVN and cAmyg, a circle tool (area=3022 pixels) was used to determine mean optical density for each section such that anatomically atlas

15   matched sections for each animal were compared in the identical region of the PVN and cAmyg. The EW cell bodies expressing urocortin were too diffuse to analyze using standard optical density methods. Therefore, parameters were used such that the computer determined the number of cells within the designated

20   EW expressing a minimum optical density by color and cell size a s predetermined to exclude non-positive cells and background silver grains. Each cell determined to be positive by the computer for urocortin mRNA was then also counted for optical density. The average optical density and cell number for each section was then

25   compared.

As illustrated in Figure 5A, urocortin mRNA was significantly increased in the rostral region of the Edinger Westphal (EW) nucleus for both the number of cells expressing (Fig. 5B) as well as in the density of urocortin

mRNA per cell (Figure 5C) in the mutant animals. The central nucleus of the amygdala (cAmyg) showed a significant increase in CRF mRNA in the null mutant animals (Figs. 5A & 5D). No significant change in CRF mRNA in the PVN was detected in basal,

5   nonstressed animals (Figure 5A & 5E). The expression patterns or levels of CRFR1 mRNA in the brain or anterior lobe of the pituitary gland did not differ between the mutant and wild type mice (data not shown). These results show that CRFR2 mutant mice have increased expression levels of CRF mRNA in the cAmyg and

10 urocortin mRNA in the rostral Edinger Westphal nucleus.

EXAMPLE 11

Evaluation of hypotension in response to UCN in CRFR2 Mutant Mice

15   Previous reports have shown hypotension in response to a peripheral injection of urocortin (2). Additionally, CRFR2s have been localized to the vascular endothelial cells (5, 8) and have been hypothesized to be responsible for the vasodilatory action of urocortin. In order to test this hypothesis, CRFR2 mutant

20 and control mice were injected with urocortin and the alteration in their blood pressure was measured.

            The cardiovascular responses to intravenous infusion of urocortin and sodium nitroprusside, a vasodilator, were examined in mice (wild type: n = 5; mutant: n = 3) anesthetized

25 with isofluorine. The arterial catheter for blood pressure recording was fabricated from a sterile PE-10 tubing softened and pulled to an outer diameter of ~0.4 mm. The femoral artery was exposed, and the arterial   catheter filled with heparin saline (500 U/ml) was implanted   and secured with surgical threads

and tissue glue (Vetbond). The catheter was connected to a blood pressure transducer (Statham), and the arterial pressure pulses were displayed on a Gould pen-recorder. A second catheter was then implanted in the external jugular vein for intravenous infusion of drugs. Drug infusion was performed 30 min following completion of the cannulation procedure. The venous catheter was connected to a drug-filled syringe. Infusion was completed within 0.5-1.0 min. Both wild type and mutants received an identical dose of urocortin (0.1 µg in 200 µl of 0.9% saline) and saline (as a control).

The doses used were determined from preliminary experiments with reference to data obtained from corresponding studies in Sprague Dawley rats (2). In order to verify that the lack of cardiovascular response to the urocortin injection in mutants was not attributed to the loss of ability of the mice to vasodilate, the mutant mice also received a second infusion of sodium nitroprusside (0.8 µg in 100 µl of 0.9% saline) following recovery of arterial pressure from the urocortin infusion. The mean arterial pressure (MAP) was determined from the blood pressure tracings.

Intravenous infusion of urocortin (0.1 µg) resulted in a prominent depressor response (-28.3 ± 2.0 mm Hg) in control mice (Fig. 6). The reduction in arterial pressure persisted throughout the recording period (90-120 min). In stark contrast, the mutants showed no measurable responses to urocortin[only 1 mutant mouse examined showed a very small and transient reduction (-3.5 mm Hg) in arterial pressure which is likely attributable to the injection pressure itself (Fig. 6)]. In order to verify that the peripheral vasculature of the mutants was able to vasodilate

in response to another stimulus, sodium nitroprusside (NP), which causes vasodilation as a nitric oxide donor, was administered to mutant mice. A rapid and robust depressor response was consistently observed in response to the sodium nitroprusside

5   injection (-30.0 ± 5.0 mm Hg) as shown in Figure 6 (white bar). Basal MAP under anesthesia was not significantly different between the mutant (76 mm Hg) and wild type mice (74 mm Hg) during this experiment.

10

EXAMPLE 12

CRFR2 affects vascularization

Histological analysis of organs from the CRFR2 mutant mice revealed that vascularization of several organs including the

15   brain, the pituitary, and adrenal glands is markedly increased in the CRFR deficient mice (data not shown). This indicates that CRFR2 may act as a direct regulator of vascularization. Ligands serving as agonists or antagonists of CRFR2 can thus be used for manipulation of vascularization in disease states. One example is

20   cancer where increased vascularization is essential for the growth of cancer cells. By stimulating CRFR2 activity, an agonist can be used to inhibit vascularization and thus hinder the growth of cancer cells.

25

EXAMPLE 13

Summary of effects of CRFR2 deletion on anxiety and stress

The results presented here suggest that the CRFR2 null mutant mice display a stress-sensitive and

29

anxiety-like phenotype. Although basal feeding and weight gain were normal, mutant mice responded to food deprivation by consuming less food during the refeeding period following the stress of food deprivation. While this may be an effect of

5   metabolism, as the mutant and control animals show no differences in body weight gain or loss during the experiment, it is possible that the stress of food deprivation alters the anxiety state of the animal, thus decreasing their appetite or affecting their metabolism. The mutant mice also displayed a rapid HPA

10   response to restraint stress, again suggesting that these animals are more sensitive to stress. The decrease in ACTH levels in the mutants observed following ten minutes of restraint may be the result of a more rapid negative glucocorticoid feedback on the hypothalamus, since the mutant mice showed higher steroid levels

15   earlier than the control mice. Therefore, the possibility of a second mechanism leading to activation of the adrenal gland in the mutant mice cannot be ruled out. Taken together, the feeding and HPA axis results suggest a hypersensitivity to stress in the CRFR2 mutant mice, although other physiological explanations may be

20   possibly involved in either the altered feeding response or the increased rate in which the HPA axis in the mutant mice responds to stress.

The mutant mice also displayed increased anxiety-like behavior in the EPM and open field tests. However, these mice

25   show similar levels of anxiety-like behavior in the light/dark box. Although pharmacological sensitivity and specificity has generally been demonstrated across many animal tests of anxiety, task differences are sometimes observed (17, 18). Performance in the light/dark paradigm may differ in that this task

correlates more with responses to novelty (neophobia) than with exploration (19) while performance in the EPM is determined by exploration of aversive environments (19). Light conditions during testing can also significantly influence the ability to detect

5   anxiolytic or anxiogenic effects in animal tests (17). This profile of results for the CRFR2 mutant mice demonstrates heightened emotionality related to exploration of aversive environments but not neophobia. The results obtained with the CRFR2 mutant mice suggest that these differences in behavioral tests may explain

10   differences in anxiety-like behavior detected in the mice.

EXAMPLE 14

Possible effects of increased CRF in cAmyg on anxiety

15   Increased CRF mRNA in the cAmyg may explain the anxiety-like behavior and increased HPA axis sensitivity of the mutant mice, since this nucleus expresses CRFR1 (8) and plays a major role in transduction of stress signals (20). In addition, the septum, which contains an abundance of CRFR2, has been shown to

20   modulate the activity of the amygdala (21-23) and lesions of this nucleus result in decreased ACTH secretion following restraint stress (24-27). Lesions of the amygdala have been shown to block CRF-induced anxiety (20) as well as hyperemotionality resulting from septal lesions (21). This neural pathway may explain the

25   decreased anxiety-like behavior seen in the CRFR1 deficient mice (10) as well as the increased anxiety-like behavior in the CRFR2 deficient mice. Therefore, the CRFR2 mutant mouse provides possible evidence for a   novel   mechanism   of   receptor modulation in anxiety-like   behavior. It is possible that during

stress CRFR2 in the lateral septum modulates activity of the amygdala, and in the absence of CRFR2, unimpeded amygdala activity may result in increased anxiety-like behavior.

CRFR2 in the lateral septum may also function as an inhibitor of PVN actions on HPA responses to stress. Since the mutant mice are deficient for CRFR2 in the lateral septum, stress-induced activation of the PVN may occur more rapidly. Further, CRFR2 is the predominant CRF receptor expressed in the PVN in unstressed animals, whereas CRFR1 is found in the PVN only under stress conditions (28, 29). Therefore, in the absence of CRFR2 during stress local effects on PVN activity may be altered.

EXAMPLE 15

Possible mechanisms for anxiety caused by increased UCN mRNA in the rostral EW

Increased urocortin mRNA in the rostral EW may be a second mechanism leading to increased anxiety-like behavior in the mutant mice, since urocortin has been shown to induce anxiety-like behaviors when injected intravenously (30). Additional explanations for the increased anxiety-like behavior, such as heightened sensitivity of the autonomic nervous system (31-33), cannot yet be ruled out. Previous studies using antisense oligonucleotides have found conflicting results regarding the role of CRFR2 in anxiety and behavior, although these reports did show an anxiolytic-like effect by injection of CRFR1 antisense oligonucleotides (34,35).

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EXAMPLE 16

CRFR2 Null Mice and the sensitivity of the autonomic nervous
system

        Additional explanations for the increased anxiety-like
5   behavior, such as heightened sensitivity of the autonomic nervous
system (31-33), cannot yet be ruled out. Previous studies using
antisense oligonucleotides have found conflicting results regarding
the role of CRFR2 in anxiety and behavior (34, 35). Although
these reports show an anxiolytic-like effect by injection of CRFR1
10 antisense oligonucleotides, neither study reported consistent
findings regarding injection of the CRFR2 antisense
oligonucleotides. While the technique of antisense oligonucleotide
injection offers potential promise, it remains under scrutiny since
decreased levels of protein cannot be substituted for complete
15 elimination of the target, as is accomplished in a knock-out animal.

EXAMPLE 17

20 Effect of UCN on vasodilation

        Absence of CRFR2 in the null mutant mice allowed for
confirmation of the effect of urocortin on vasodilation. Mutant
mice had no response to intravenous urocortin, while wild type
animals showed a dramatic decrease in mean arteriole pressure.
25 Injection of nitroprusside resulted in vasodilation in the mutants,
thus confirming that the lack of response to urocortin was not due
to a physical inability of the mutant vasculature to dilate, but
specifically to the absence   of CRFR2. These results support
the hypothesis that the   effect of urocortin on hypotension

(2, 16) occurs via action at CRFR2 in the vascular endothelial cells (5, 8), since the CRFR2 mutant mice showed no response to urocortin. Although the physiological stimulus under which UCN-induced vasodilation would most likely occur is not currently known, the effect of urocortin on CRFR2 in the vasculature may be an interesting target in drug development for hypertension.

Summary

In summary, these results demonstrate that CRFR2 deficient mice exhibit increased anxiety-like behavior and a hypersensitive HPA axis in response to stress. CRFR1 and CRFR2 mutant mice provide valuable models of anxiety and depression and may further help delineate the molecular mechanisms underlying these diseases. Study of the CRF signaling pathway and its role in the management of anxiety and depression may provide the necessary clues required for the effective treatment of these diseases.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1.   A   non-natural   transgenic   mouse   with   a
disruption in at least one allele of the corticotropin releasing factor
5   receptor   2   (CRFR2)   such   that   said   mouse   does   not   express
corticotropin releasing factor receptor 2 protein from said allele.

2.   The transgenic mouse of claim 1, wherein the
DNA   sequences   for   exons   10,   11,   and   12   of   said   corticotropin
10   releasing factor receptor 2 allele have been deleted.

3.   The transgenic mouse of claim 2, wherein said
DNA   sequences   have   been   replaced   with   a   neomycin   resistance
gene   cassette.
15

4.   The transgenic mouse of claim 3, wherein said
mouse is heterozygous for said replacement.

5.   The transgenic mouse of claim 3, wherein said
20   mouse is homozygous for said replacement.

6.   The progeny of a mating between a mouse of
claim 3 and a mouse of another strain.

25   7.   A method of screening a compound for anxiety
modulating activity, comprising the steps of:
a)   administering said compound to the transgenic
mouse of claim 5;
b)   testing   said   mouse   for   anxiety-related

37

behavior; and,

      c)   comparing anxiety-like behavior of said mouse with anxiety-like behavior in a second transgenic mouse of claim 5 to which said compound was not administered.

5

      8.   The method of claim 7, wherein said mice are tested for anxiety using a test paradigm selected from the group consisting of an elevated plus maze, a light/dark box, and an open field test..

10

      9.   A method of screening a compound for depression-modulating activity, comprising the steps of:

      a).   administering said compound to the transgenic mouse of claim 5;

15   b).   testing said mouse for depression-like behavior; and,

      c).   comparing depression-like behavior of said mouse with depression-like behavior in a second transgenic mouse of claim 5 to which said compound was not administered.

20

      10.   A method of screening for compounds which control blood pressure, comprising the steps of:

      a).   administering a compound to the transgenic mouse of claim 5;

25   b).   testing said transgenic mouse for alterations in blood pressure; and,

      c).   comparing alterations of blood pressure in said transgenic mouse with alterations of blood pressure in a second mouse, wherein said second mouse is selected from

the group consisting of a transgenic mouse of claim 5 to which said compound was not administered and a wild type mouse to which said compound was also administered.

5   11.   A method of screening for compounds which affect angiogenesis, comprising the steps of:

a).   administering a compound to the transgenic mouse of claim 5;

b).   assaying said transgenic mouse for alterations in

10   angiogenesis; and,

c).   comparing alterations of angiogenesis in said transgenic mouse with alterations of angiogenesis in mice selected from the group consisting of transgenic mice of claim 5 to which said compound was not administered and wild type mice to which

15   said compound was administered.

12.   A method of screening a compound for effects on the response of the hypothalamic-pituitary-adrenal axis to stress, comprising the steps of:

20   a).   administering said compound to the transgenic mouse of claim 5;

b).   placing said mouse in a stress-inducing situation,

c).   monitoring plasma levels of corticosterone and adrenocorticotropic hormone in said mouse; and,

25   d).   comparing said levels to those in a transgenic mouse of claim 5 not placed in said stress-inducing situation.

13.   The   method of claim 12, wherein said stress-inducing   situation   is physical restraint-stress.

14.   A method of determining the effects of CRFR2 on a second protein, comprising the steps of

   a).   administering an agonist that affects the second protein to the transgenic mouse of claim 5;

5   b)   performing an assay of the second protein, wherein said assay is selected from the group consisting of assays of protein expression and assays of protein activity; and,

   c).   comparing assay results on said transgenic mouse with those obtained from a wild type mouse administered

10   the same agonist.

15.   The method of claim 14, wherein said second protein is selected from the group consisting of corticotropin releasing factor, corticotropin releasing factor receptor 1,

15   urocortin, corticotropin receptors and urocortin receptors.

16.   The transgenic mouse of claim 5 used for the study corticotropin releasing factor receptor 1 responses, wherein said responses are unhindered by the presence of CRFR2.

20

17.   A method of increasing vascularization in a target tissues or an individual comprising the step of admininstering a CRFR2 inhibitor to said tissue or individual.

25   18.   A method of inhibiting vascularization in a target tissue or individual comprising the step of admininstering an agonist which stimulates CRFR2 activity.

19.   The   method of claim 18, wherein said

method is used to treat cancer.

Fig. 1A

Genotype +/+   -/-   -/-   +/+   -/-   +/-

4.4kb
3.7kb
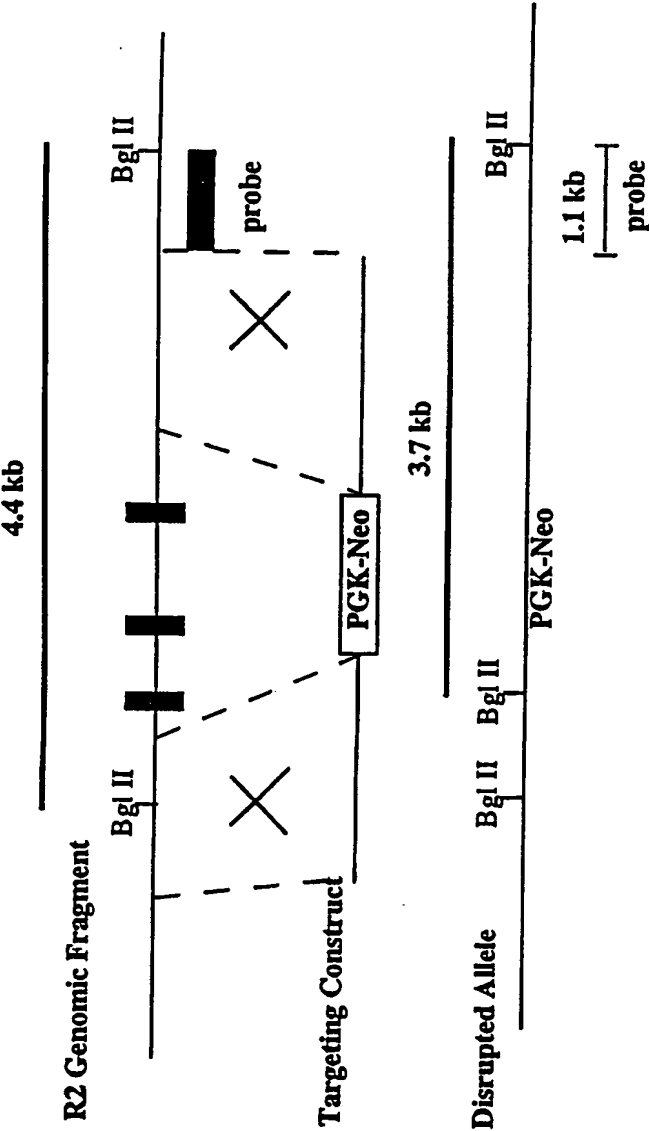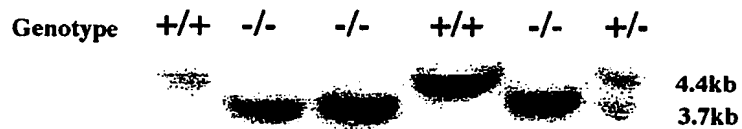
Fig. 1B
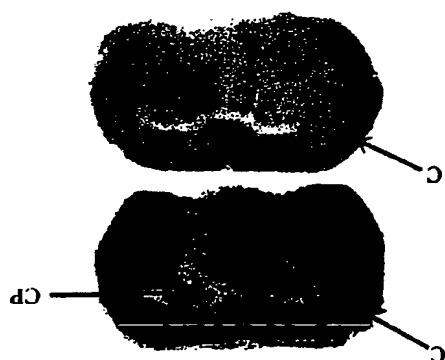
Fig. 1C
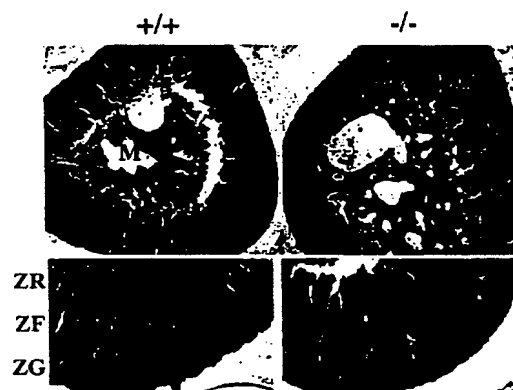
Fig. 1D
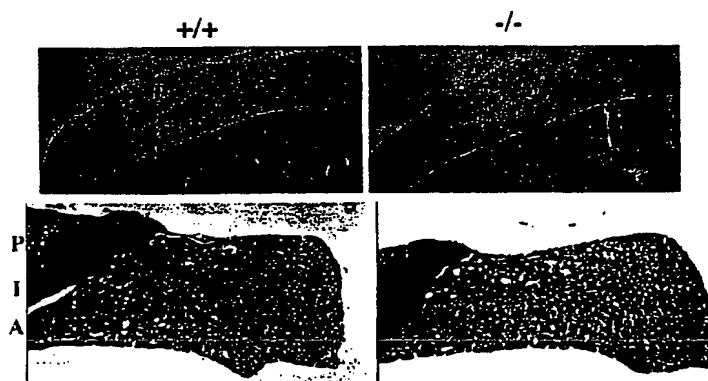
Fig. 1E
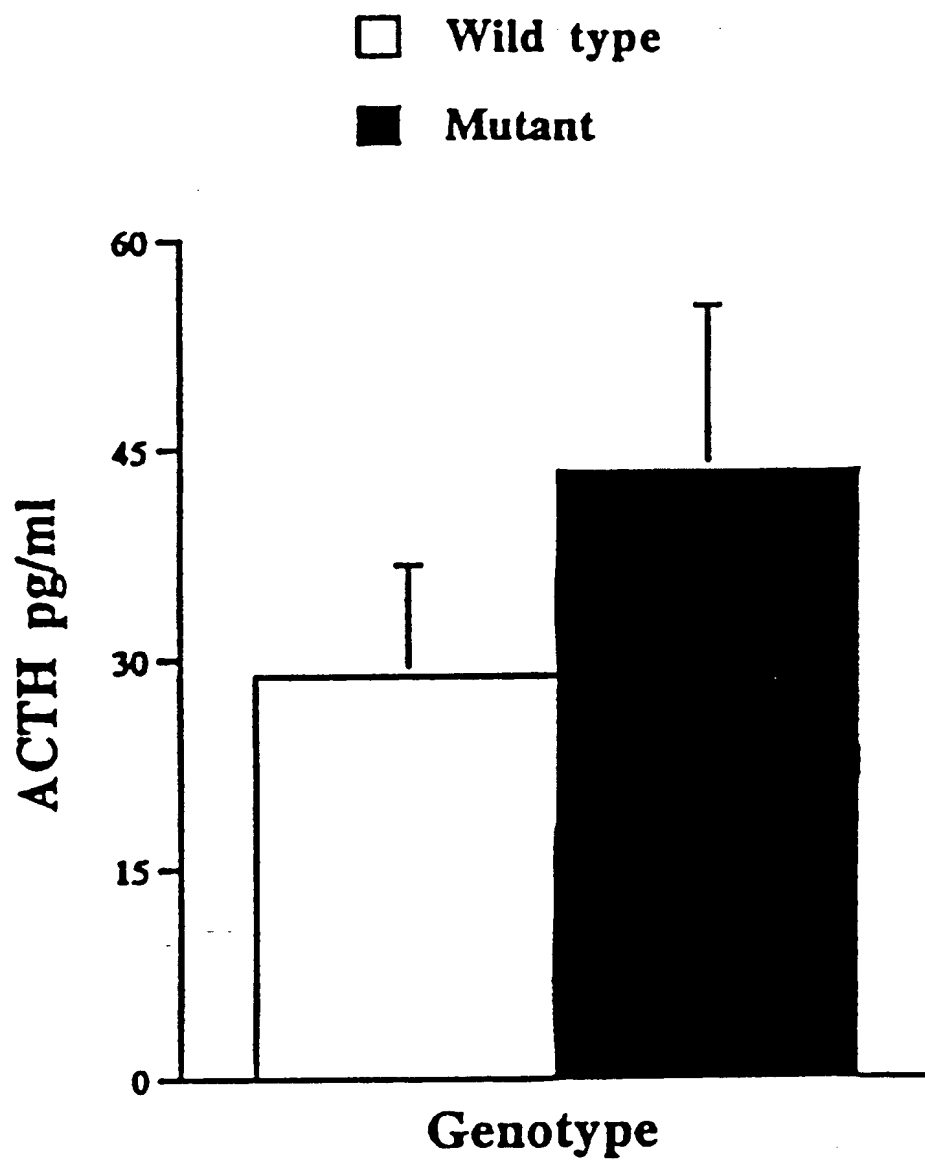
Fig. 2A
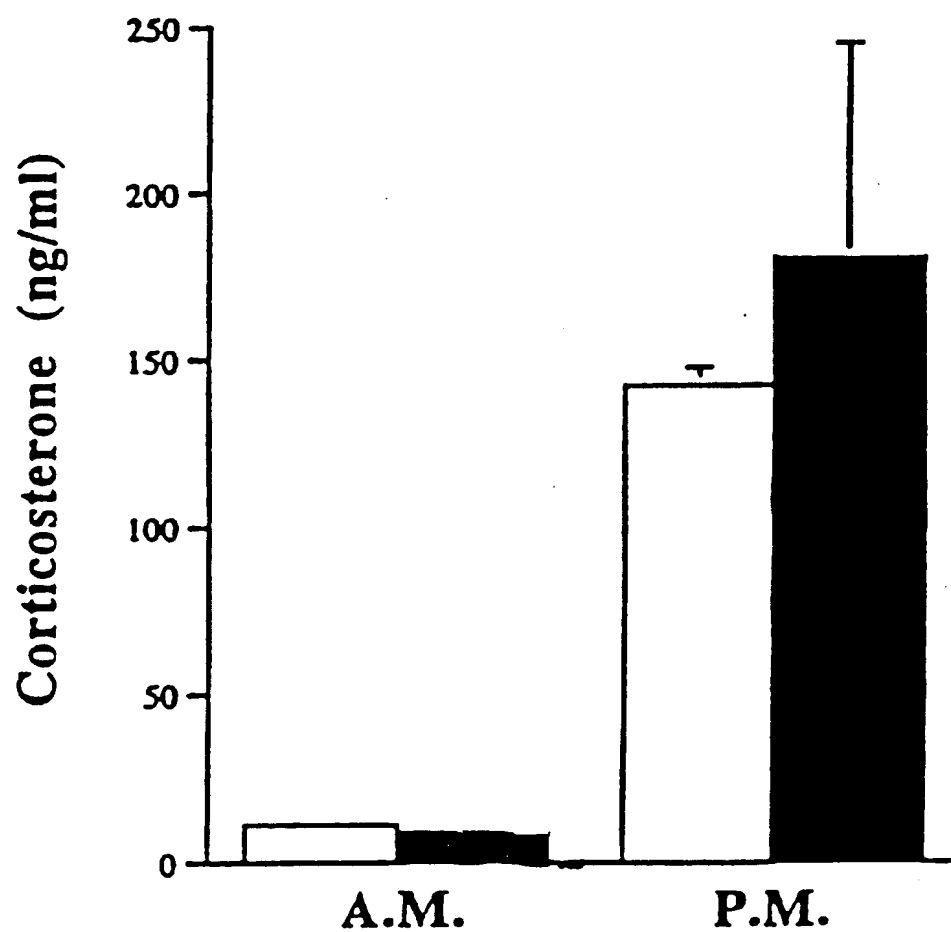
Fig. 2B
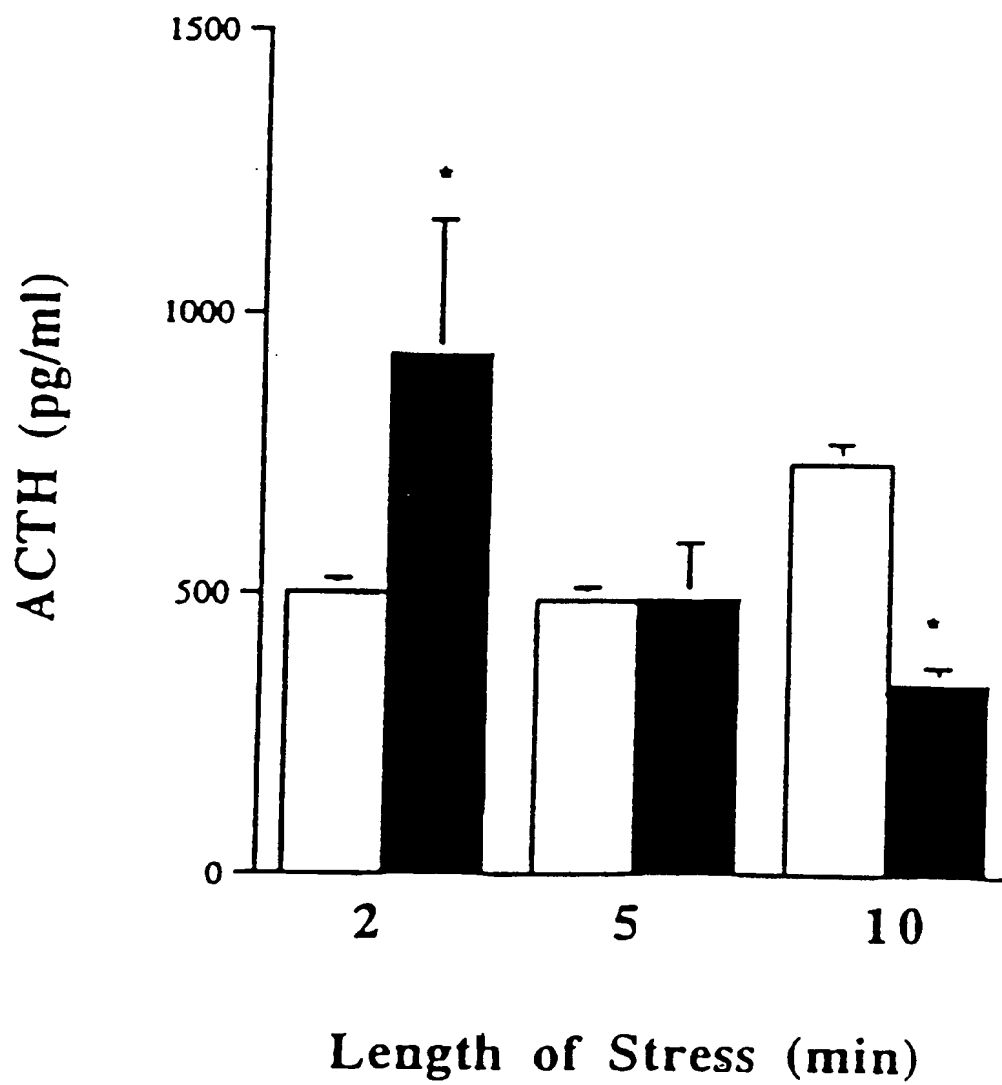
Fig. 2C
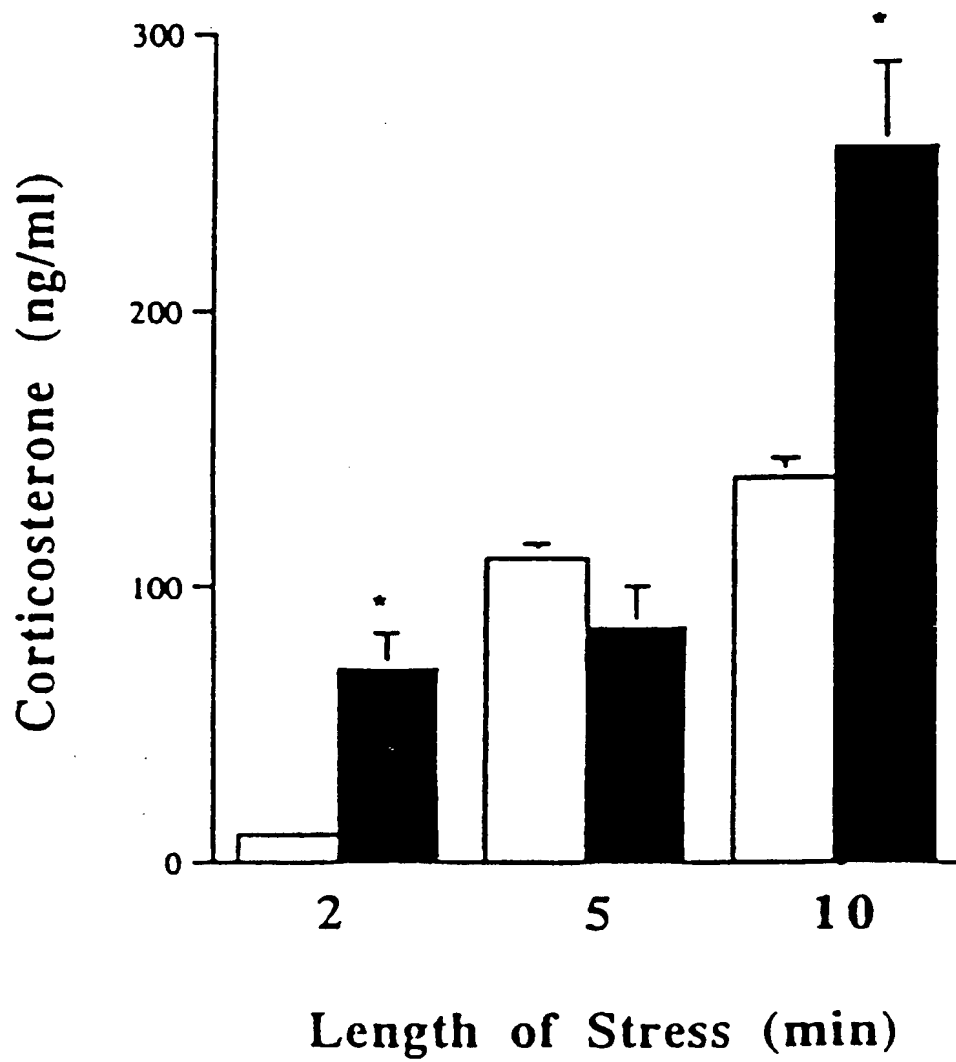
Fig. 2D
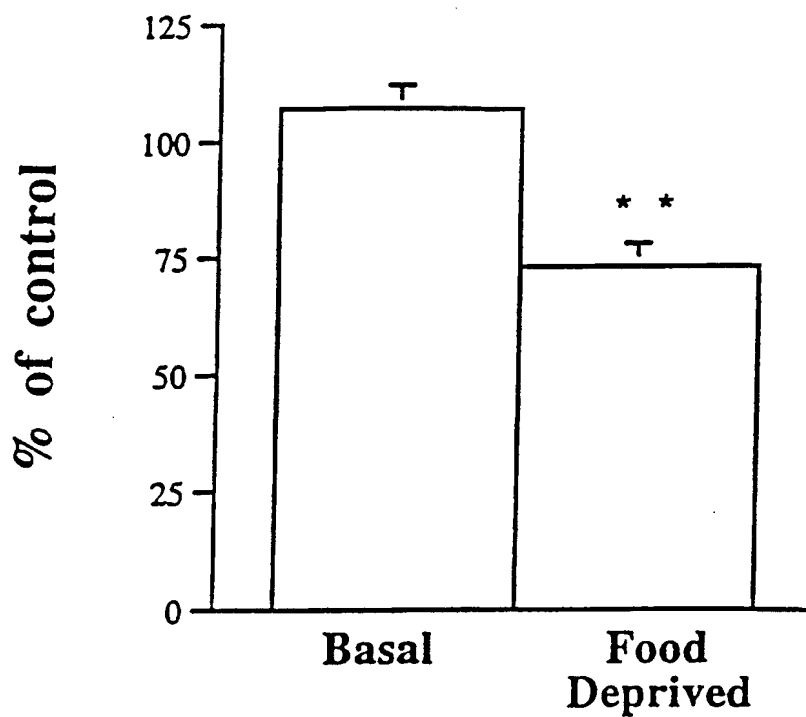
Fig. 3A
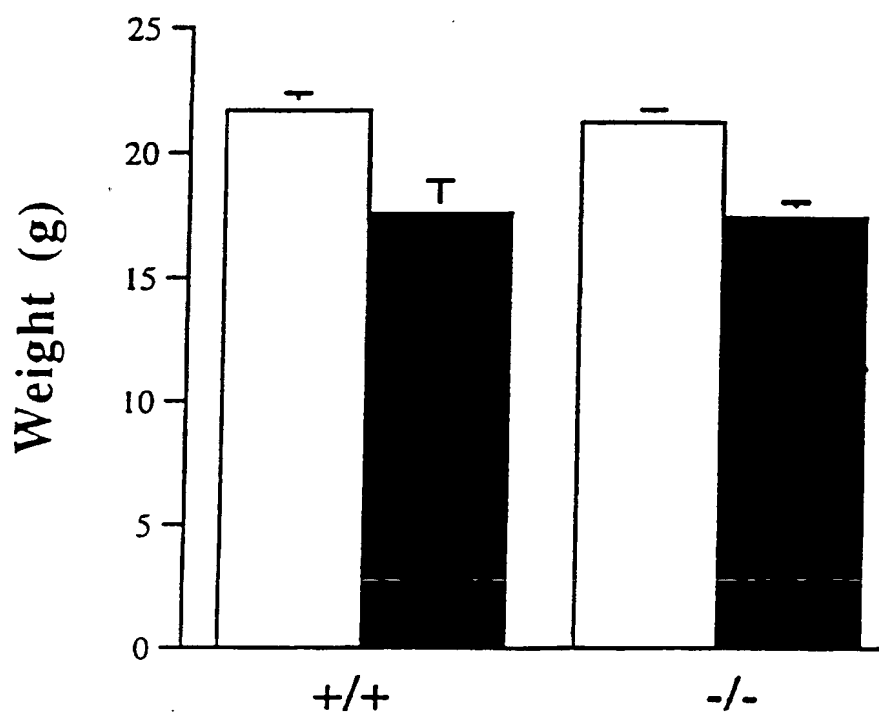
Fig. 3B
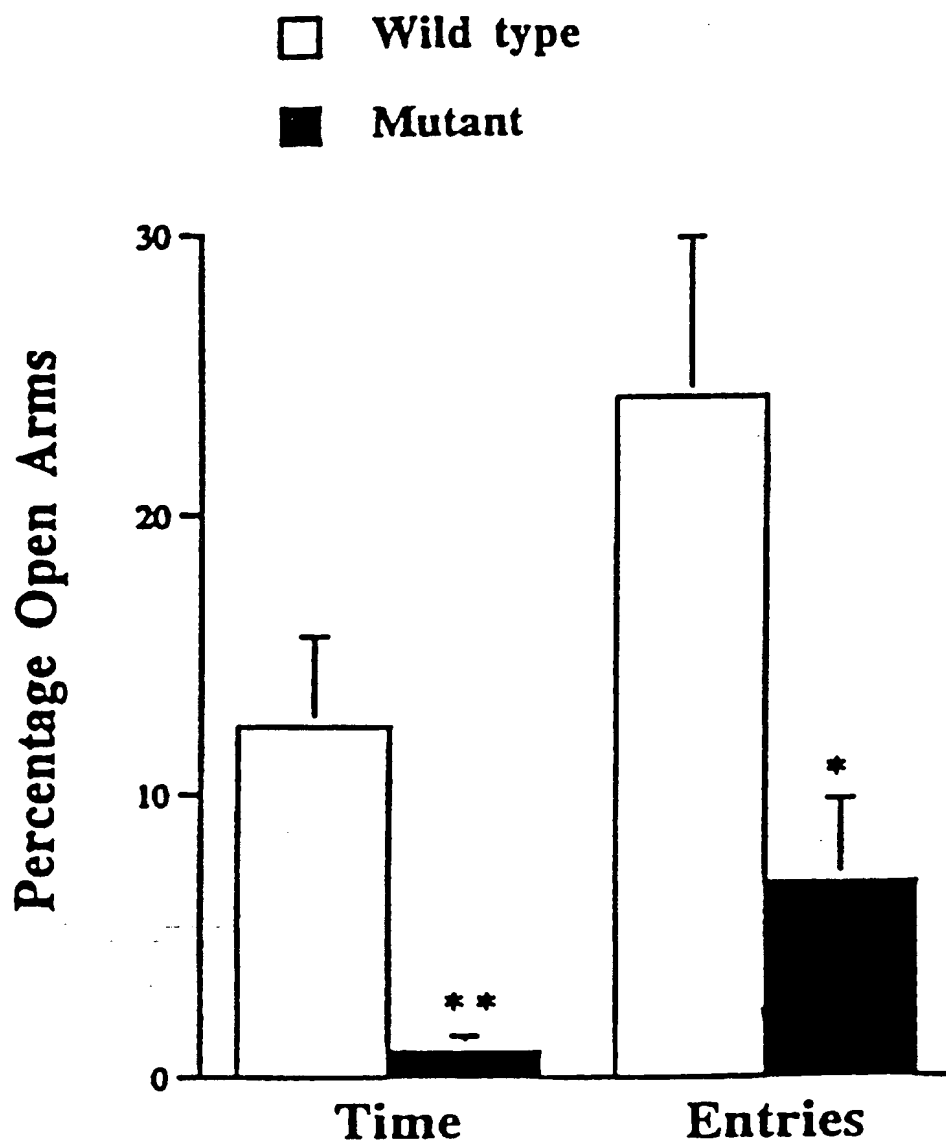
Fig. 4A

Fig. 4B

Fig. 4C
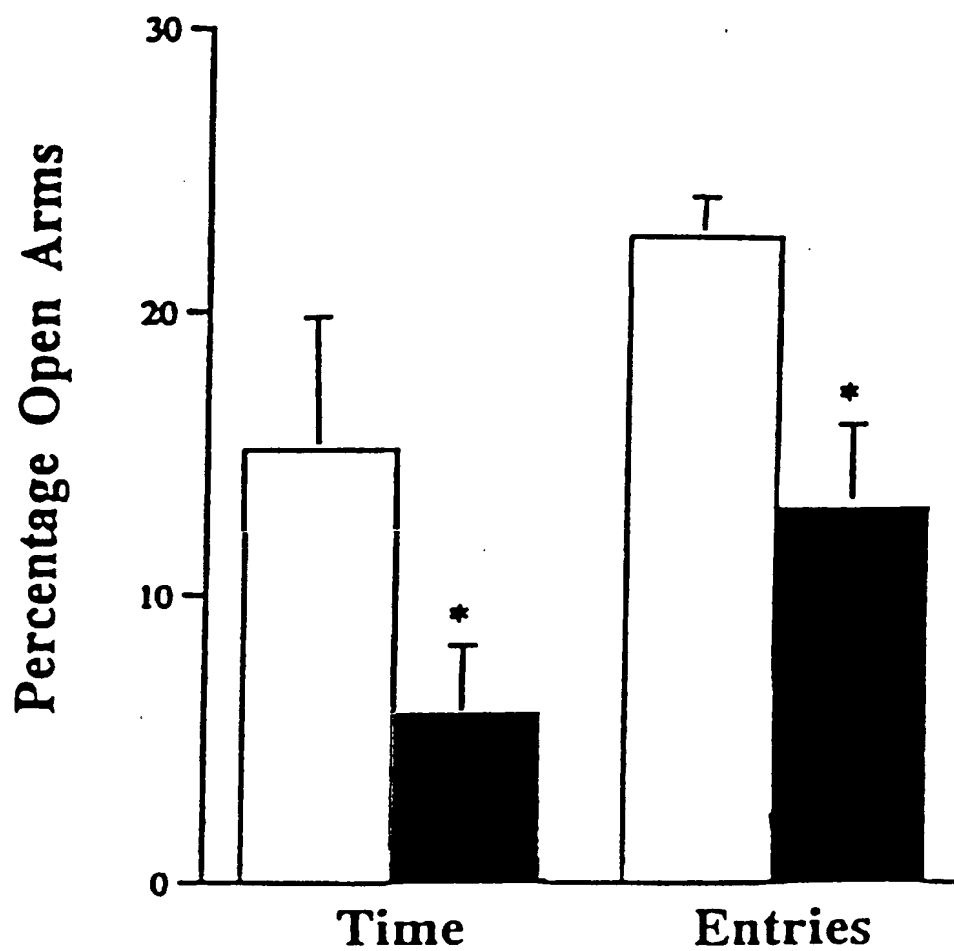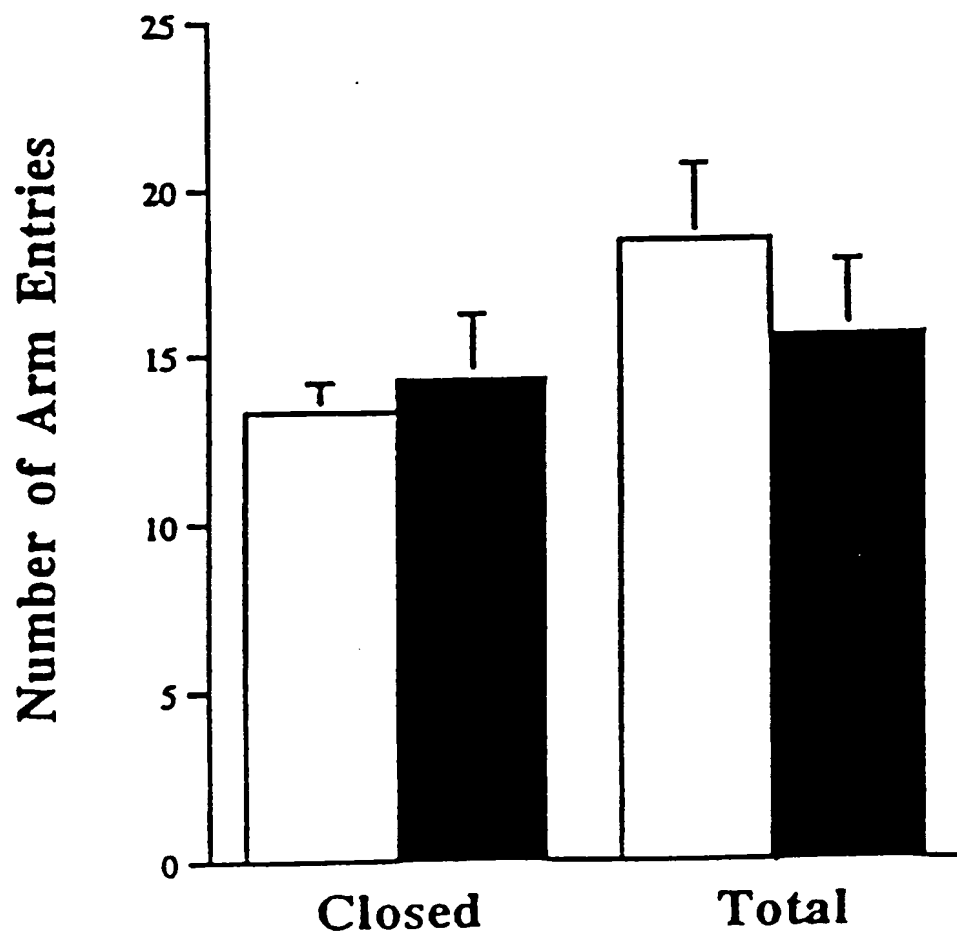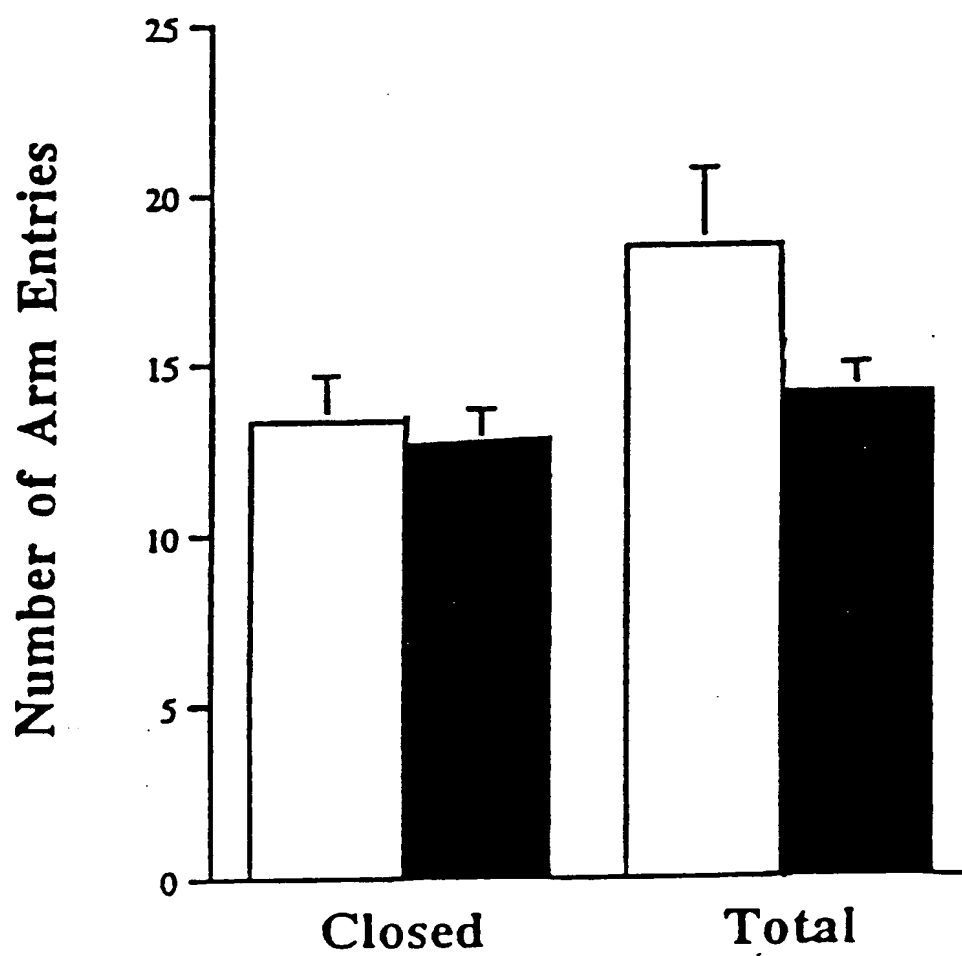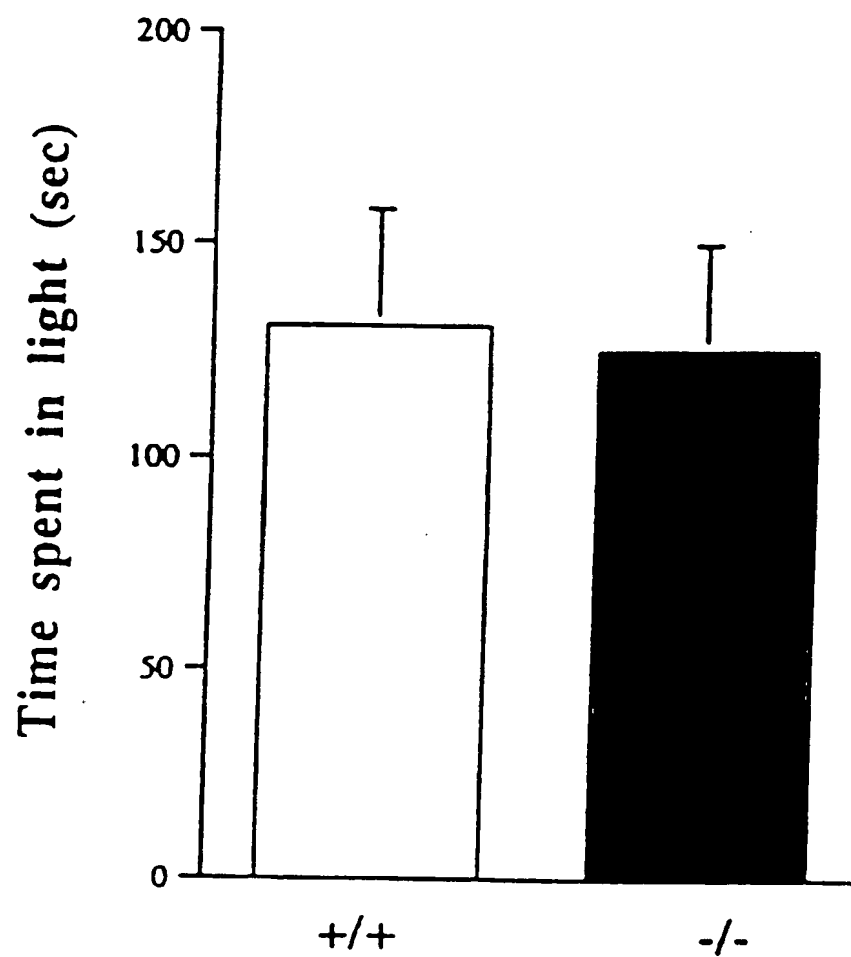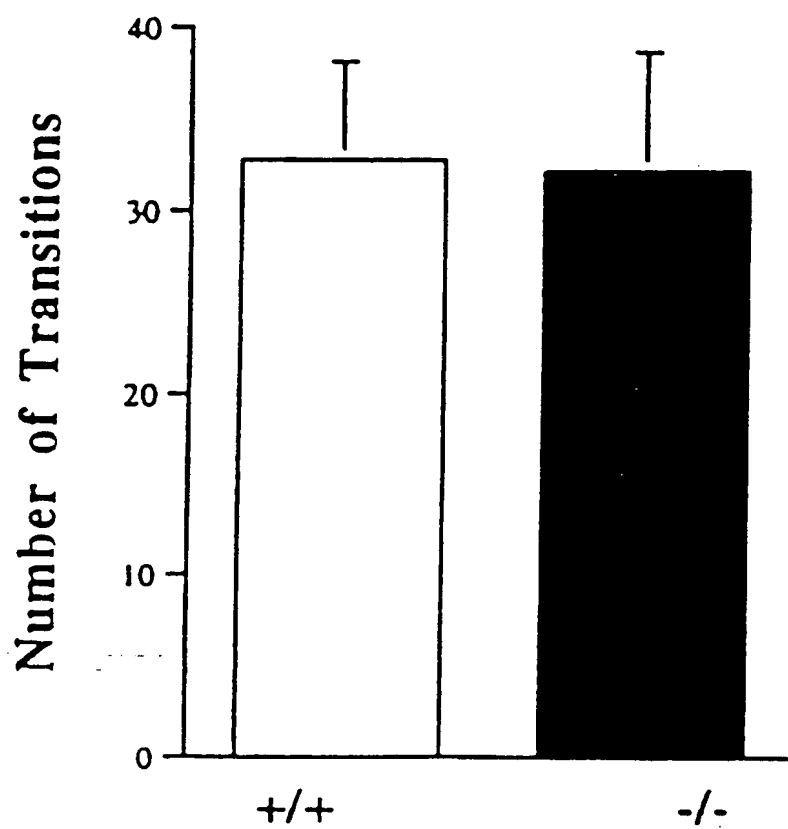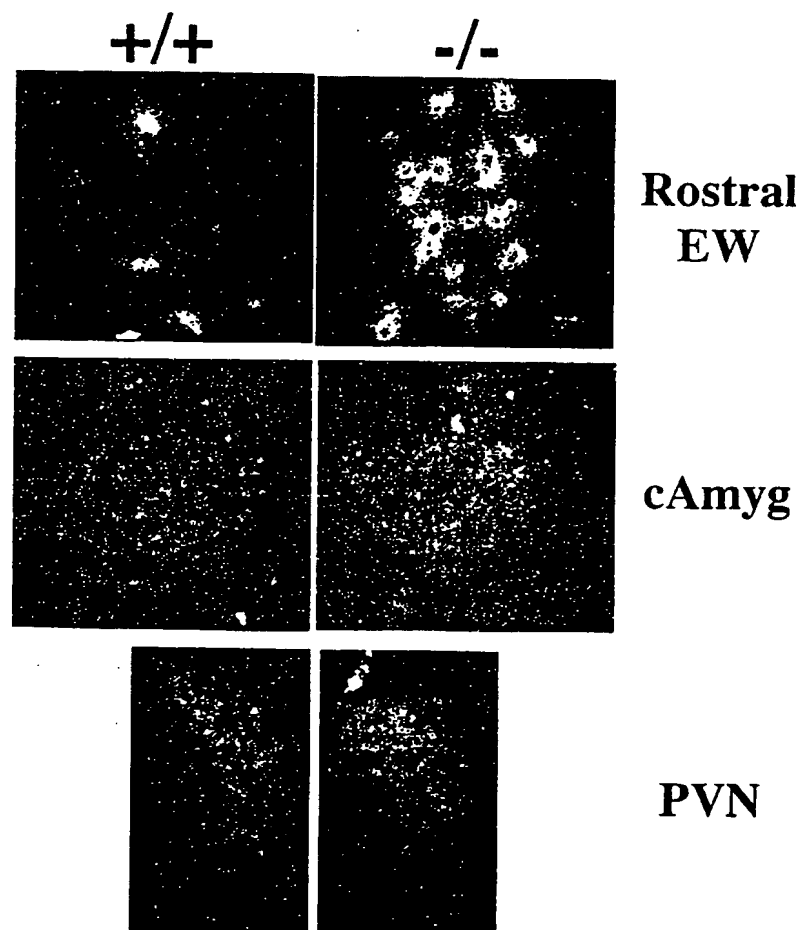
Fig. 4D

Fig. 4E

Fig. 4F

Fig. 4G

Fig. 4H

+/+   **-/-**

Rostral
EW

cAmyg

PVN
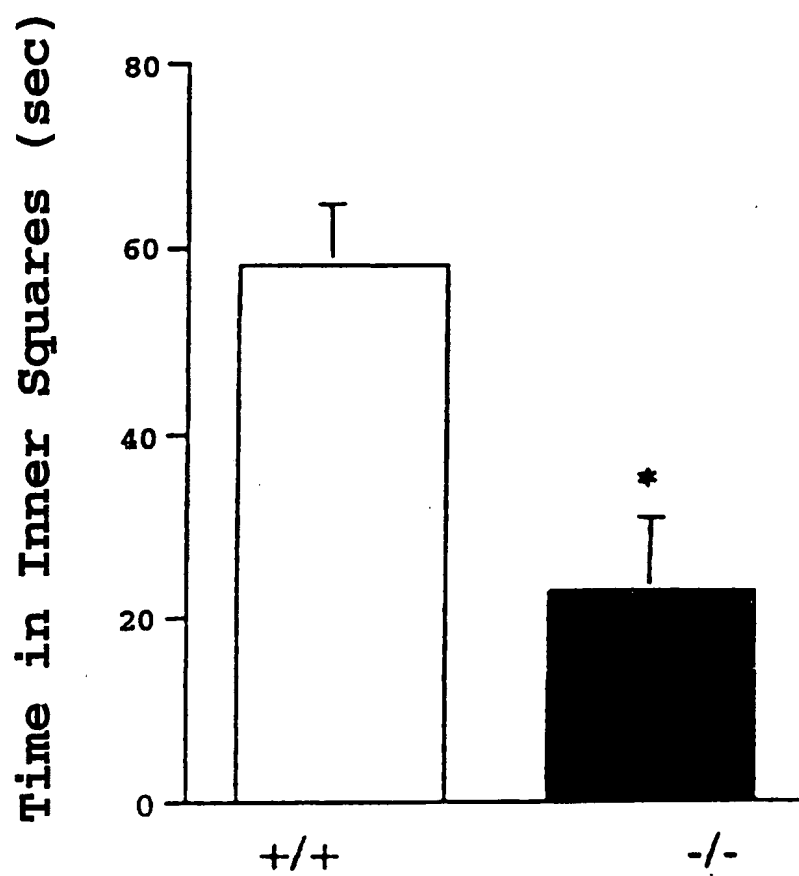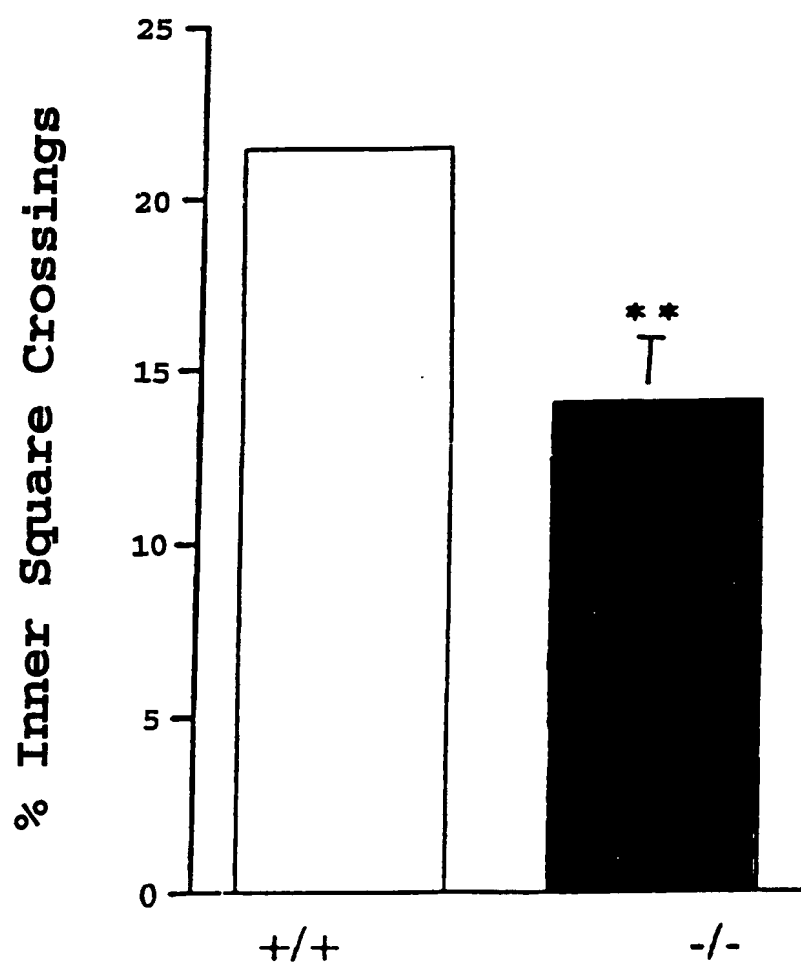
Fig. 5A
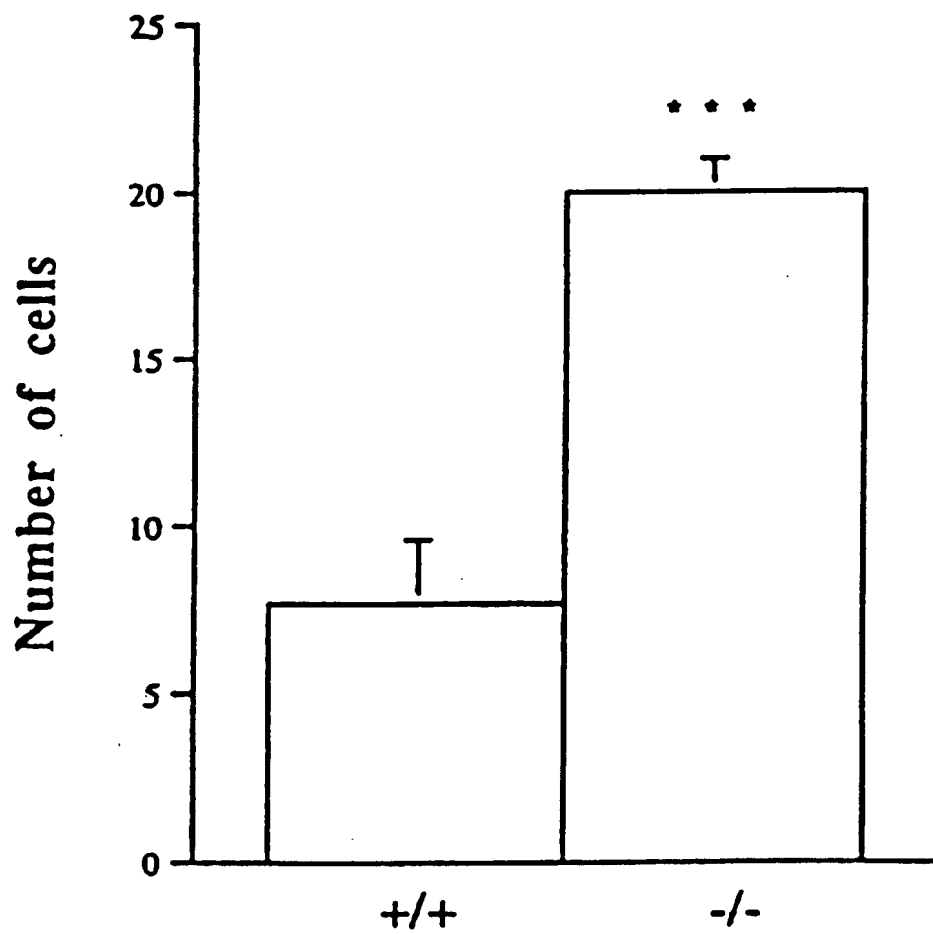
Fig. 5B
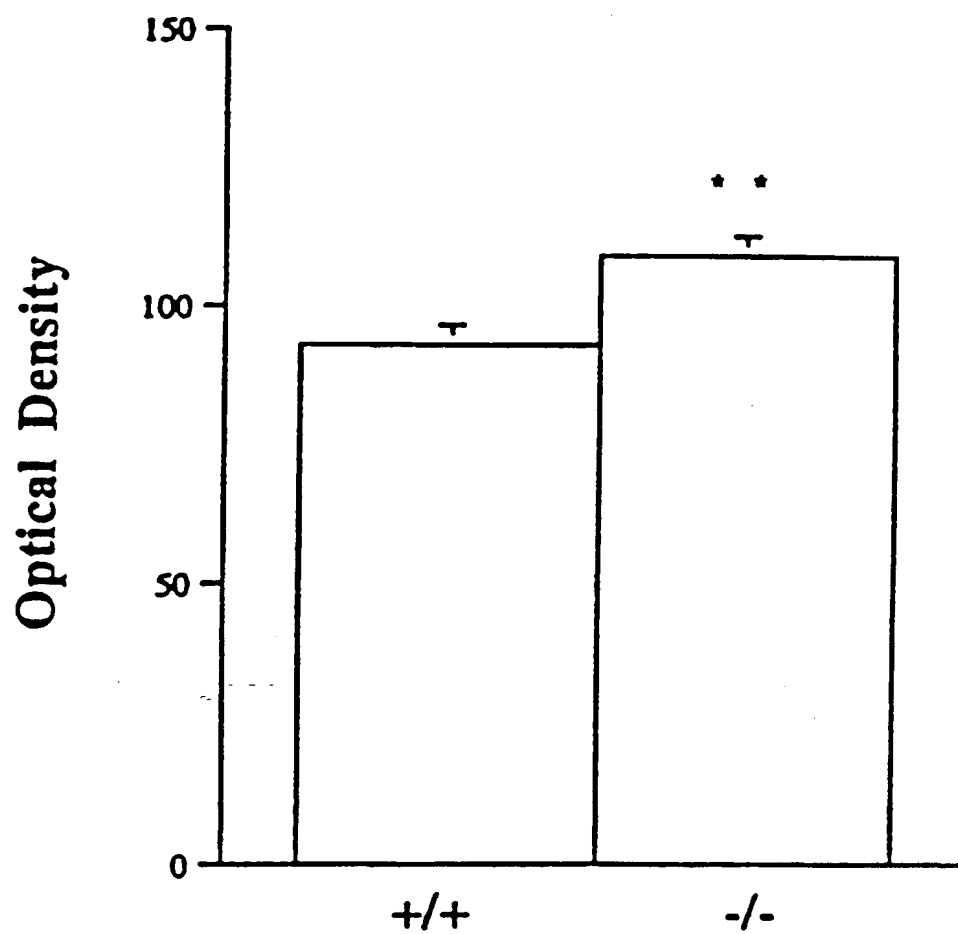
Fig. 5C
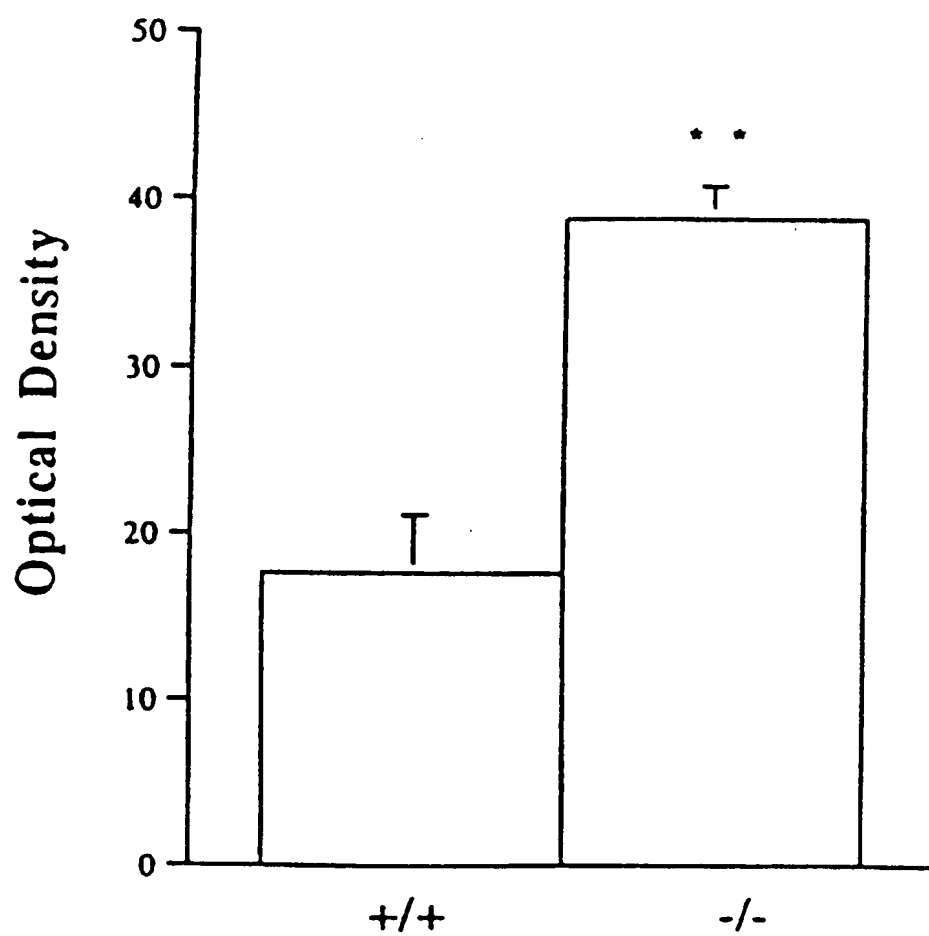
Fig. 5D
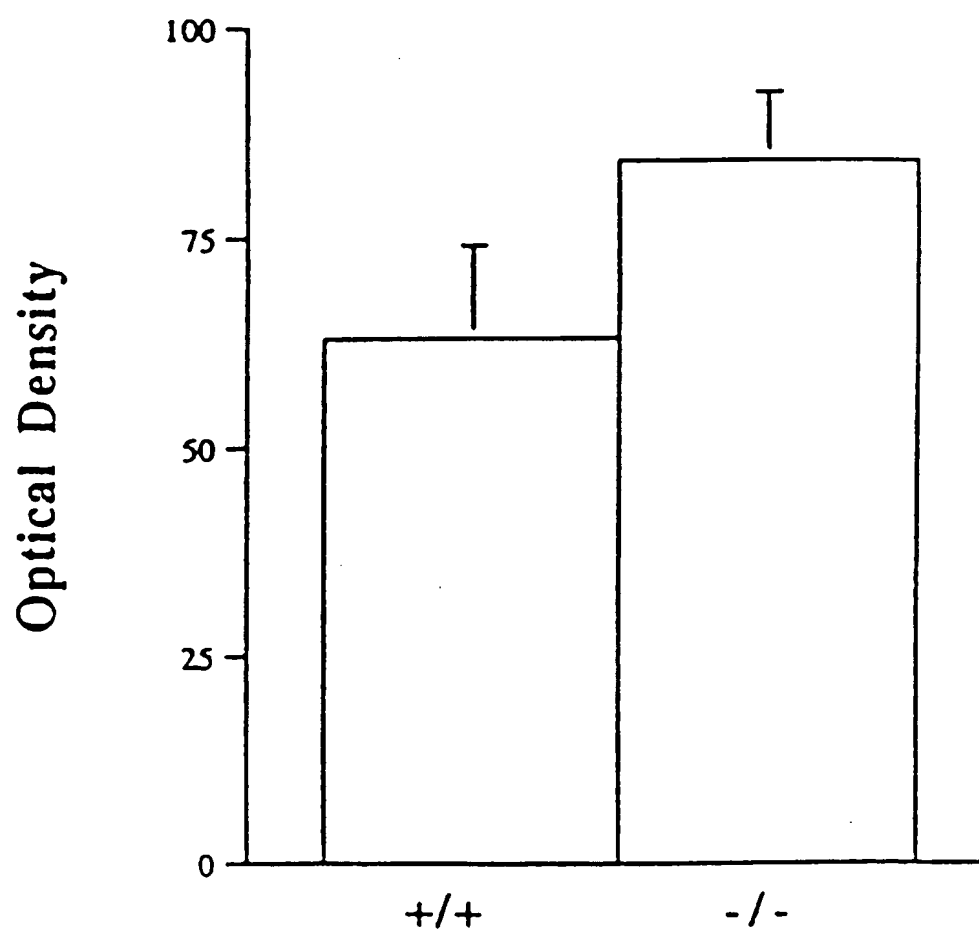
Fig. 5E

Change in MAP (mm Hg)
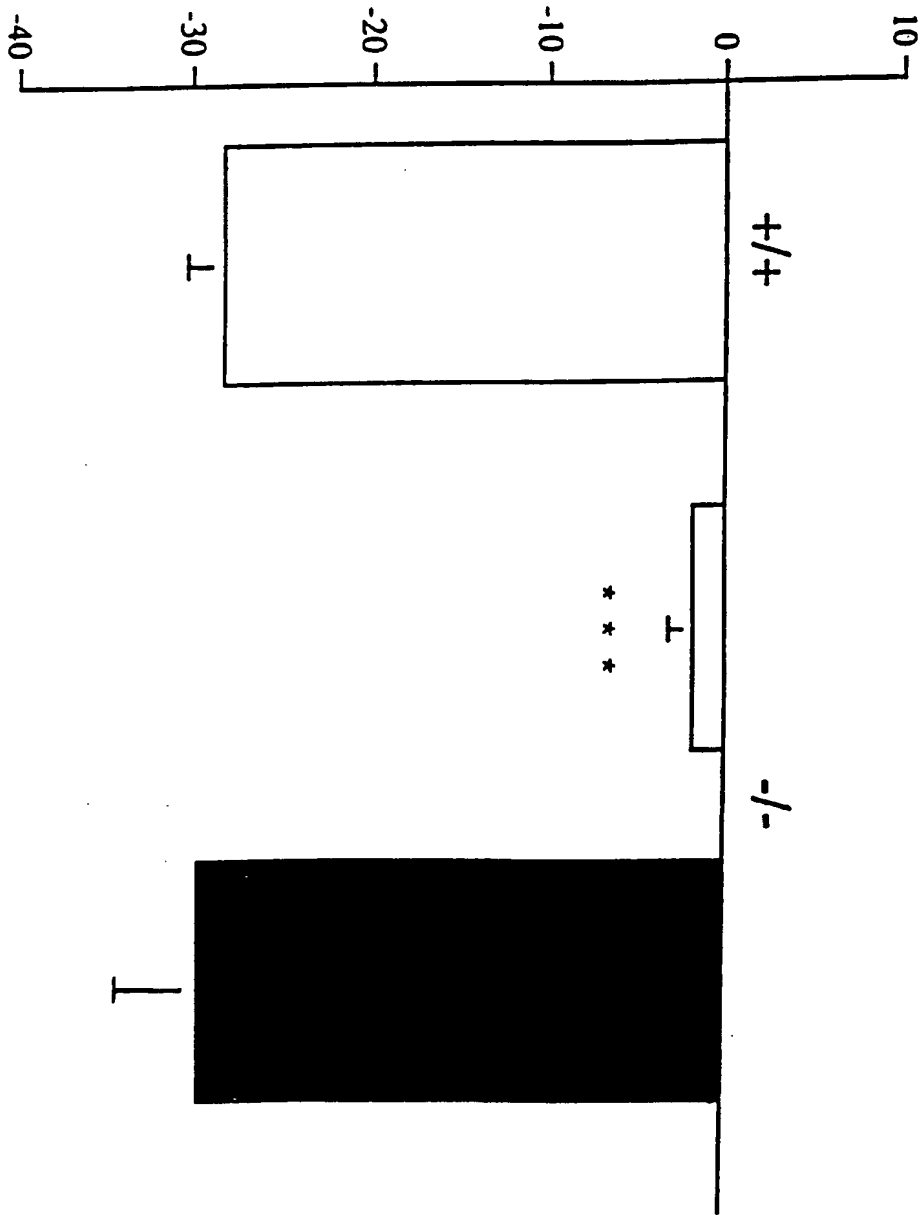
Fig. 6

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A01K 67/027
US CL :800/18
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 800/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN: MEDLINE CAPLUS BIOTECHDS EMBASE BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	COSTE et al. Abnormal adaptations to stress and impaired cardiovascular function in mice lacking corticotropin releasing hormone receptor-2. Nature Genetics, April 2000, Vol. 24, No. 4, pages 403-409, see abstract.	1-19
A, P	RADULOVIC et al, Modulation of learning and anxiety by corticotropin-releasing factor (CRF) and stress. J. Neurosci. 15 June, 1999, Vol 19, No. 12, pages 5016-5025, see abstract.	1-19

[X] Further documents are listed in the continuation of Box C.   [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 AUGUST 2000	01 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer RICHARD SCHNIZER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet) (July 1998)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIAW et al, Localization of agonist- and antagonist-binding domains of human corticotropin-releaing factor receptors. Mol. Endocrinol. December, 1997, Vol. 11, No. 13, pages 2048-2053, see abstract.	1-19
A	RUHMANN et al . Sructural requirements fro peptidic antagonists of teh corticotropin-releasing factor receptor (CRFR). Proc. Nat. Acad. Sci. USA. 1996, Vol. 95, No. 26, pages 15264-15269, see abstract.	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/19371

Box I   Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                          ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1)) (July 1998)★

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-16, drawn to a transgenic mouse and methods of use.
Group II, claim(s) 17, drawn to a method of using a CRFR2 inhibitor.
Group III, claim(s) 18 and 19, drawn to a method of inhbiting vascularization using a CRFR2 agonist.
The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:
        The linking technical feature of the three groups is the CRFR2 receptor. However, the CRFR2 receptor was known in the art prior to the filing of the instant application (see Liaw et al Mol. Endocrinol. 11(13): 2048-2053, 1997), thus it cannot be a special technical feature under PCT Rule 13.2 because it does not constitute a contribution over the prior art.